CLINICAL
LABORATORY METHODS

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A MANUAL OF TECHNIQUE AND MORPHOLOGY
DESIGNED FOR THE USE OF STUDENTS
AND PRACTITIONERS OF MEDICINE

BY

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TO

WILLIAM SYDNEY THAYER

AND

GEORGE DOCK

THIS VOLUME IS DEDICATED

WITH THE AFFECTION AND GRATITUDE OF

THE AUTHOR

PREFACE

Coincident with the improvement in medical education in this country there has been a widespread increase in the use of laboratory methods as aids to diagnosis. Not only is this true in the case of the more recent graduates in medicine, but, what is more hopeful, the older practitioners—those whose college days preceded the introduction of Clinical Pathology into the medical curricula—are quite generally realizing the necessity of the laboratory in their daily work. Probably no one thing has done more to bring about this much desired result than the discovery by Wassermann and his co-workers of the well-known serum reaction for the diagnosis of syphilis; unconsciously, perhaps, but none the less effectively, attention has been focused upon laboratory diagnostic methods.

The present volume is not a text-book of Clinical Pathology; it is a manual of laboratory technique and morphology, dealing merely with methods and with morphological elements which are of diagnostic importance. It attempts to give in detail the means of detecting the abnormal in urine, gastric contents, feces, blood, sputum, and puncture fluids. Unlike the text-books, the significance of the abnormal is not discussed.

That there is need for such a work the author has long believed. There is much which it is absolutely essential that the student of medicine—graduate and undergradute—remember. He must know, for example, under what conditions albuminuria may occur, whether it be of nephritic, cardiac, toxic, physiologic, or whatever origin. He must be aware of the possible significance of a secondary anemia, of an atypical reduction test in the urine, of Charcot-Leyden crystals in sputa, of a hydrochloric acid deficit in the gastric contents. But it is useless to try to burden the memory with the details of the various laboratory methods, by which such abnormalities are detected, and with the sources of error in the methods.

PREFACE

No attempt has been made to include within the present volume a multiplicity of methods; in fact, the aim of author has been to select one method or more of proved value. Nor have the more exact, time-consuming methods of physiological chemistry been drawn upon; in his daily work the average clinician has not the time, if he has the ability, to employ them.

Free use has been made of the following works: Emerson's "Clinical Diagnosis," Wood's "Chemical and Microscopical Diagnosis," Simon's "Clinical Diagnosis," Sahli's "Klinische Untersuchungsmethoden," Hoppe-Seyler's "Handbuch der chemischen Analyse," Hammarsten's "Lehrbuch der physiologischen Chemie," Neubauer-Huppert's "Analyse des Harns," Schmidt and Strasburger's "Die Faezes des Menschen," Braun's "Thierische Parasiten des Menschen," Blanchard's "Traité de Zoologie Médicale," Cabot's "Clinical Pathology of the Blood," Naegeli's "Blutkrankheiten und Blutdiagnostik," and Türk's "Vorlesungen ueber klinische Haematologie." Other authors have been consulted less freely. To the more recent literature direct reference has been given in the form of footnotes; in all instances the writer has endeavored to give proper credit to authors.

It is hoped that the volume will prove helpful to medical students who have completed a course in Clinical Pathology and to practitioners of medicine, or that it may serve as a supplement to a course of laboratory lectures.

For the original illustrations in black and white the author is indebted to Dr. James S. Brotherhood. It is a pleasure, also, to acknowledge his indebtedness to his wife and to his mother for assistance in the preparation of the index and in other ways.

ROGER SYLVESTER MORRIS.

St. Louis.

CONTENTS

CHAPTER I

THE URINE

CHAPTER II

THE GASTRIC JUICE

CHAPTER III

THE FECES

CHAPTER IV

THE SPUTUM

CHAPTER V

THE BLOOD

CHAPTER VI

PUNCTURE FLUIDS

LIST OF ILLUSTRATIONS

CLINICAL LABORATORY METHODS

CHAPTER I

THE URINE

Collection of the Urine.—For chemical examination, as a general rule, the total amount of urine for the twenty-four hours should be preserved. The reason for saving *all* the urine is that different voidings may vary greatly in their chemical composition. In the morning, for example, an albuminuric may excrete urine which is normal chemically, whereas specimens obtained after the patient has had more or less exercise may contain albumin. Thus, it becomes necessary that a mixture of all the urine passed during the twenty-four hours be obtained in order to avoid the possibility of error, for the examination of the early morning specimen alone, in the case just cited, would be entirely misleading. Special circumstances arise at times, nevertheless, which make it desirable to break the rule and to secure one or more voidings at special hours of the day. For the purpose of quantitative chemical analysis, it is, of course, absolutely essential to have the total amount of urine for twenty-four hours collected.

For *microscopic examination* a perfectly fresh specimen should always be insisted upon. The organized elements of a urinary sediment rapidly deteriorate, especially with high temperatures, so that within a few hours after the urine has been passed they may be unrecognizable, or completely disintegrated. When it is not possible to make an

immediate examination a preservative (thymol, toluol) should be added to the specimen, which is kept in an ice-chest as a further safeguard.

Preservation of the Urine.—To all twelve or twenty-four-hour specimens of urine a preservative should be added to prevent decomposition through bacterial growth. The urine should also be kept on ice when possible. The receptacle for the urine must be perfectly cleaned and tightly stoppered.

(1) *Toluol* (toluene) is, on the whole, one of the most satisfactory preservatives. It apparently interferes with none of the urinary tests. Bacterial growth is successfully inhibited by means of it. The one disadvantage in its use results from the fact that toluol floats on the urine; it is necessary to pipette or siphon the urine to obtain it without admixture of the preservative. The objection is a minor one. Diacetic acid may be preserved for weeks, whereas it disappears in a short time when other preservatives are used. In acidosis, therefore, toluol should be used as the preservative. Organized sediments are often beautifully preserved, but it must be remembered that casts or cells, becoming attached to droplets of toluol, rise to the surface and may be missed, when only a few are present; spontaneous sedimentation cannot be relied on if toluol is employed. The pipette used for withdrawing the sediment must be wiped to remove the toluol before placing the drop on a slide for examination.

(2) *Chloroform* is the most generally used preservative for chemical work. It is a fairly strong reducing agent, and urine preserved with it must be boiled to drive it off before any of the reduction tests for sugar are performed. If the sediment is to be examined, care should be exercised to avoid drawing up chloroform with it.

(3) *Thymol* is very satisfactory, and with it the formed elements of the urine are often very well preserved. As Weinberger [1] has shown, many urines to which thymol has been added give a positive Heller's test, though albumin be absent, a source of error which must be kept in mind. A positive test for bile may also be obtained after thymol preservation (Emerson).

Gum camphor and *formaldehyde* are used occasionally as preservatives. Formaldehyde, like chloroform, is a reducing agent. When available, toluol, chloroform, or thymol is to be preferred.

Macroscopic Examination of the Urine.—As a general rule, normal, freshly voided urine is perfectly clear; the same is true of the majority of pathological urines. Occasionally, if the reaction of the urine be alkaline when voided, a turbidity may result from the precipitation of the phosphates and carbonates in the bladder, in the absence of a cystitis. Ordinarily, however, fresh urine, when cloudy or turbid, contains pathological ingredients, such as blood, pus, bacteria in large number, phosphates, etc. Normal and pathological urines will become turbid and produce a macroscopic deposit, more or less abundant, if allowed to stand for some hours. Concentrated urine often furnishes an abundant precipitate of urates on cooling; the urates may be redissolved by warming the specimen. More frequently bacterial decomposition is the cause of the turbidity.

The *nubecula* is a translucent cloud, composed chiefly of mucin (mucous threads) enmeshing epithelial or other cells, which forms in the urine a short time after it is passed.

[1] Weinberger, W. "Thymol as a source of error in Heller's test for urinary protein." *Jour. A. M. A.*, 1909, LII, 1310.

The *color* of the urine is usually dependent on the quantity of water excreted in the twenty-four hours; the smaller the amount of urine the deeper the color, and *vice versa.* Normal urinary pigments in increased concentration or pathological pigments may lead to abnormal coloration of the urine (see urobilin, bilirubin, hemoglobin, hematoporphyrin, etc.). Following the administration of certain drugs, the color of the urine may be altered, the most striking change being the green color produced by methylene blue.

Quantity of Urine.—The normal average amount of urine for the twenty-four hours in this country is about 900 to 1,200 c. c. The limits of the normal are said to be 800 to 3,000 c. c. (Emerson). In health the quantity depends chiefly upon two factors, the amount of water consumed and the amount lost by perspiration. In disease the quantity of urine passed in twenty-four hours may be normal, increased, decreased, or nil.

In certain diseases the urine is saved to advantage in twelve-hour periods, 7 A. M. to 7 P. M., and 7 P. M. to 7 A. M. In health the ratio of the quantity of the day urine is to that of the night urine as 67:33, though it may be as 50:50, considering the total amount for twenty-four hours as 100. In disease the quantity voided during the night may exceed that for the day, as Edmunds [1] and others have shown.

REACTION OF URINE

The reaction of the urine is usually slightly *acid,* owing to the presence of an excess of dihydrogen (diacid) phosphates. An *amphoteric* reaction (red litmus turned blue and blue turned red) may be encountered, due to the fact

[1] Edmunds, C. W. "Observations on the quantity of day and night urine." *N. Y. Med. Jour.,* 1904, LXXIX, 245.

that monosodium phosphate, an acid salt, may exist in the urine in conjunction with disodium phosphate, which is alkaline. An *alkaline* reaction is produced largely by an excess of alkaline phosphates and carbonates. That the salts are not the only factor in rendering a urine acid has been shown by Folin, who finds that at times nearly half of the acidity may be due to organic acids.

Litmus paper is used in testing the reaction of the urine. Unpreserved specimens, which have been allowed to stand for some time before testing, are often alkaline from ammoniacal fermentation produced by bacteria. The alkalinity in this case is differentiated from that due to fixed alkali by the odor, by the fact that on boiling the specimen the steam will turn blue a piece of moistened red litmus held in the neck of the test tube, or will cause a white frost of ammonium chlorid to appear on a glass rod, which has been dipped in hydrochloric acid. In disease the urine may be ammoniacal before it is voided.

Quantitative Determination of Urinary Acidity

For quantitative determination of the acidity the twenty-four-hour specimen is used. It is necessary to prevent decomposition by the addition of a preservative.

Folin's Method.[1]

Reagents:

$\frac{N}{10}$ sodium hydrate.[2]

0.5 per cent. phenolphthalein in 50 per cent. alcohol.

Potassium oxalate, neutral.

[1] Folin, O. "The acidity of the urine." *Amer. Jour. Physiol.*, 1903, IX, 265.

[2] A normal solution of acid or alkali should be purchased from a reliable firm. With this as a standard, the physician may easily prepare most of the remaining normal solutions required in routine work.

Method.—"With a pipette transfer 25 c. c. of urine into a small Erlenmeyer flask (capacity 200 c. c.). Add one or, at most, two drops of phenophthalein and 15 to 20 gms. powdered potassium oxalate. Shake about one minute and titrate *at once* with tenth normal hydrate until a faint, yet distinct, pink coloration is produced throughout the contents of the flask. Shaking should be continued during the titration, so as to keep the solution as strong as possible in oxalate." The number of cubic centimeters of sodium hydrate used multiplied by 4 gives the acidity per cent. in terms of tenth normal alkali.

The inaccuracy of direct titration of the urine with sodium hydrate, as proposed by Naegeli, is pointed out by Folin. The two chief sources of error are ammonium salts and the occurrence of calcium in the presence of acid phosphates. By first treating the urine with potassium oxalate each of these sources of error is practically eliminated.

Normal values with this method are 25 to 30 acidity per cent. (Wood).

SPECIFIC GRAVITY

As a rule, the determination of the specific gravity of the urine is of real value only in the twenty-four-hour specimen. It is usually determined by means of an urinometer. The short, small instruments designed for the purpose of taking the specific gravity of small quantities of urine are usually very inaccurate.

In using the urinometer the urine is carefully poured into a glass cylinder, so that no foam is produced. Should foam collect despite the precautions, even though there be only a few bubbles, they should be removed with filter paper. The cylinder must be sufficiently wide to permit the urinometer to float freely without coming in contact with

its wall. The reading is made with the eye on a level with the *bottom* of the meniscus (the concave upper surface of the fluid). The instruments are standardized for use at a temperature of 15° C. ordinarily. For each 3° C. above this temperature the specific gravity is depressed one point in the third decimal place. As an example, if the specific gravity of a urine were found to be 1.015 at 24° C., the corrected reading would be 1.015+0.003=1.018.

In case the specimen of urine furnished for examination be small, the urine which remains after the necessary tests have been performed may be diluted with water and the specific gravity of the diluted specimen determined. The last two figures of the specific gravity found are multiplied by the dilution; the result approximates the specific gravity of the undiluted urine.

Normally the specific gravity of the twenty-four-hour specimen varies between about 1.010 and 1.025; absolute limits for the normal cannot be assigned, for so many factors enter into the determination of the specific gravity that in individual instances the figures given may be passed in either direction, without necessarily signifying disease. It must be remembered that readings made with the urinometer are not absolutely correct, but are sufficiently accurate for clinical purposes. Where greater accuracy is required a pycnometer should be employed.

An approximate idea of the amount of solids dissolved in the urine may be obtained by multiplying the last two figures of the specific gravity by 2.33 (Häser's coefficient), the result being the amount of solids in grams.

UREA

The normal amount of urea excreted daily in the urine varies within rather wide limits. Values between 20 and

40 gms. are usually found. In clinical work urea determinations have been practically abandoned, except in the diagnosis of unilateral renal disease.

Hüfner's Hypobromite Method.—The most convenient apparatus for applying this test is Heinz's modification of the Doremus tube. It consists of a J-shaped tube mounted on a stand. A bulb is blown in the extreme end of the "tail" of the J-tube and a second tube of 2 c. c. capacity, graduated in $\frac{1}{10}$ c. c., is blown into the upright arm of the J-shaped tube. The connection between the two tubes may be cut by means of a glass cock in the 2 c. c. tube. The upper end of the J-tube is sealed.

The reagent, Rice's bromin solution, is prepared as follows:

Sol. 1.	Sodium hydrate	40.0 gm.
	Distilled water	100.0 c. c.
Sol. 2.	Bromin	10.0 c. c.
	Potassium bromid	10.0 gm.
	Distilled water	80.0 c. c.

The two solutions are kept in separate bottles, and at the time of performing the test are mixed in equal volumes.

Method.—Fill the small tube with the urine. The stop-cock is then opened until the urine reaches the zero mark. The excess of urine, which has run into the large J-tube, is removed from the latter by washing it with water, the upper end of the tube containing the urine being appropriately sealed to prevent its escape. The J-tube is now filled with the mixed solutions, sufficient of the latter being employed to completely fill the upright (all air must be displaced). The stop-cock is opened and the urine is *slowly* run into the mixed solutions. As the two fluids come in

contact, the hypobromite liberates nitrogen gas, which collects at the upper end of the large tube. The volume of gas liberated by 1 c. c. of urine is read on the scale marked on the upright arm of the J-tube, and gives the urea in grams in 1 c. c. of urine.

The method is very inaccurate as a means for determination of urea; the results obtained approach more nearly the total nitrogen of the urine. For this reason the method is inapplicable, where exact values for urea are required, as, for example, in metabolism experiments. In the diagnosis of surgical affections of the kidney, where the urine from each kidney is examined separately, marked differences in the two kidneys are shown with sufficient accuracy, and it is in this connection that the method is used most at the present time.

URIC ACID

The normal quantity of uric acid in the urine in twenty-four hours lies between 0.1 and 1.25 gm., with a patient on a mixed diet. The endogenous uric acid of the urine varies between 0.1 and 0.4 gm.

Qualitative Determination of Uric Acid

Qualitative determination of uric acid is made by the *murexid test*. A small drop of the urinary sediment or other material to be tested is dissolved in two or three drops of nitric acid in a porcelain evaporating dish. The material is evaporated to dryness, preferably on a water bath, care being exercised to avoid burning the preparation. The stain which remains on the dish has a reddish color. (A yellow stain may indicate that an insufficient quantity of nitric acid was used.) The addition of ammo-

nium hydrate or, better still, exposing the stain to ammonia fumes changes the color to a purplish red, which fades on heating. The reaction is given by uric acid and by its salts. (For further qualitative tests, see urinary sediments.)

Quantitative Determination of Uric Acid

Method of Folin and Shaffer.[1]

Reagents:

Sol. 1.	Ammonium sulphate	500.0 gm.
	Uranium acetate	5.0 gm.
	Distilled water to	650.0 c. c.
	Dissolve and then add:	
	Acetic acid, 10 per cent	60.0 c. c.
	Distilled water to	1,000.0 c. c.
Sol. 2.	Ammonium sulphate	100.0 gm.
	Distilled water to	1,000.0 c. c.
Sol. 3.	$\frac{N}{20}$ potassium permanganate.	

To prepare the twentieth normal permanganate solution dissolve 1.6 gm. of potassium permanganate in one liter of distilled water. The solution is boiled or autoclaved to render it more permanent. After it has cooled to room temperature it is titrated against tenth normal oxalic acid solution [illegible] to one liter of distilled water. With a pipette [illegible] oxalic acid [illegible] diluted [illegible]

60° C. While still hot the permanganate solution is added to it from a burette, under constant stirring, until a uniform red color appears, which persists throughout the fluid for a few seconds.[1] This is the end reaction. The quantity of permanganate solution used is read off on the burette. Since the permanganate solution has been made too strong, less than 20 c. c. of it should have been required to produce the end reaction. The permanganate solution which remains is accurately measured (with volumetric flasks and pipettes, not with cylinders), and is diluted with distilled water, so that exactly 20 c. c. will give the end reaction with 10 c. c. of tenth normal oxalic acid. When kept in a dark place, tightly stoppered, the potassium permanganate solution is fairly permanent for several months. The titer should, however, be determined from time to time with the oxalic acid solution.

Example.—If 18.9 c. c. of permanganate solution gives the end reaction with 10 c. c. of $\frac{N}{10}$ oxalic acid, and the remainder of the potassium permanganate solution amounts to 960 c. c., the necessary dilution to make the permanganate solution twentieth normal is determined by the following equation: $18.9:20::960:x$. $x=1{,}015.8$. Therefore, the amount of water necessary to add would be 1,015.8—960 or 55.8 c. c.

Method.—To 300 c. c. of urine in an Erlenmeyer flask or beaker of 500 c. c. capacity or larger, add 75 c. c. of the uranium acetate reagent (sol. 1) to precipitate phosphates and other substances, which might interfere with the accuracy of the method. Both urine and reagent must be accurately measured with volumetric pipettes. Stir the mixture

[1] Early in the titration, after the addition of the first few drops of permanganate, a red color, which may persist for fifteen seconds or so, may be noted, but it quickly disappears on adding more permanganate.

well, and allow it to stand five minutes. Then filter through a double folded filter. Measure with a pipette 125 c. c. of the filtrate (this represents 100 c. c. of the urine originally used) into each of two beakers and add 5 c. c. of ammonium hydrate to each to convert the uric acid into ammonium urate. Mix well, and set aside for twenty-four hours; by the end of this time the precipitate will have settled to the bottom of the beaker. The clear, supernatant fluid is decanted, the precipitate collected on a filter (Schleicher and Schüll's No. 597) and washed with 10 per cent. ammonium sulphate, till the filtrate is almost chlorin free. (In testing for chlorids add a little nitric acid and then a few drops of dilute silver nitrate solution (10 to 15 per cent. solution); a white precipitate or cloud is formed if chlorids are present.) The filter paper is now pierced with a glass rod, and the precipitate washed into a beaker with about 100 c. c. of distilled water. Add 15 c. c. of concentrated sulphuric acid and titrate the mixture immediately, stirring constantly, until a pink color appears throughout the fluid and persists for a few seconds.

Each cubic centimeter of twentieth normal permanganate is equivalent to 3.75 mg. of uric acid. This, multiplied by the number of c. c. of permanganate used, gives the uric acid in 100 c. c. of urine, from which the total amount for twenty-four hours is readily calculated. Since ammonium urate is slightly soluble in water and somewhat more so in urine, a correction of 3 mg. should be added to the final result for each 100 c. c. of urine.

AMMONIA

Normally the urine contains 0.6 to 0.8 gm. of ammonia daily for the average adult on a mixed diet. The limits of

the normal are about 0.3 to 1.2 gm. In health the ammonia nitrogen usually amounts to 4 to 5 per cent. of the total nitrogen, when a mixed diet is taken.

Quantitative Determination of Ammonia

(1) **Folin's Method.**[1]

Reagents:

Sodium chlorid.
Anhydrous sodium carbonate.
Petroleum or toluene.
$\frac{N}{10}$ sulphuric acid; $\frac{N}{10}$ sodium hydrate.
One per cent. aqueous solution of alizarin red.

The apparatus required includes areometer cylinders (45 cm. deep and 5 cm. in diameter), a suction pump, calcium chlorid tube, doubly perforated stoppers to fit the cylinders, and tubing for connections. Folin's tube to secure thorough mixing of air and acid is a convenience, though not a necessity. The apparatus is connected as shown in the illustration (Fig. 1), the calcium chlorid tube filled with cotton being placed between the cylinders to prevent the alkaline urine being drawn over into the acid.

Method.—With a pipette, 25 c. c. of tenth normal acid are placed in cylinder B and diluted with distilled water sufficiently to cover the end of the mixing tube. Into cylinder A, 25 c. c. of the twenty-four-hour specimen of urine are measured with a pipette. To the urine are added 8 to 10 gm. of sodium chlorid, 5 to 10 c. c. of toluol or petroleum to prevent foaming (with blood or other fluid rich in protein add some methyl alcohol also), and, finally, about 1 gm.

[1] Folin, O. "Eine neue Methode zur Bestimmung des Ammoniaks im Harne und anderen thierischen Flüssigkeiten." *Ztschr. f. physiol. Chem.*, 1902-03, XXXVII, 161.

of anhydrous sodium carbonate. After the addition of the soda the cylinder is immediately stoppered and the air current started. Before entering the urine the air current may be passed through a wash bottle containing sulphuric acid to remove ammonia in the air, though usually this precaution is unnecessary.

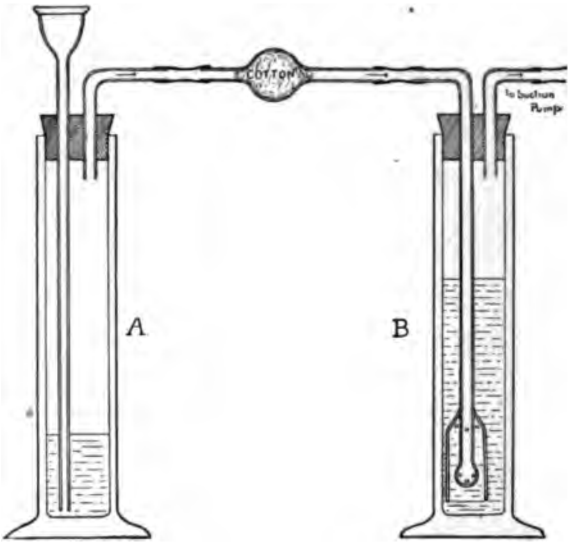


FIG. 1.—APPARATUS FOR THE QUANTITATIVE DETERMINATION OF AMMONIA ACCORDING TO FOLIN. Cylinder A for urine, cylinder B for acid.

The addition of the soda to the urine liberates the weaker base, ammonia, which is carried over by the air stream into the tenth normal sulphuric acid, by which it is neutralized. When Folin's mixing tube is used, not a trace of the ammonia escapes neutralization by the acid, even though there remains an excess of only 5 c. c. of tenth normal acid. If ordinary glass tubing be employed to pass the air through the acid, a second cylinder, containing 10 c. c. of tenth normal acid, should be interposed between the

acid cylinder and the pump to catch the ammonia which escapes neutralization.

The air pump should be capable of carrying 600 to 700 liters of air per hour through the apparatus. With a pump of this capacity working at room temperature (20 to 25° C.) all of the ammonia is carried into the acid in an hour or an hour and a half.[1]

When the process is completed the acid is poured into an Erlenmeyer flask or beaker and the cylinder rinsed with distilled water, which is added to the acid. The acid is now titrated with tenth normal sodium hydrate, using two drops of alizarin red to 200 to 300 c. c. of fluid. The end reaction is the appearance of a red color throughout the fluid; do *not* continue the titration to the appearance of a violet color. The difference between the number of cubic centimeters of acid originally taken and that of the alkali used is the number of cubic centimeters of acid neutralized by ammonia. Since one cubic centimeter of tenth normal acid is equivalent to 0.0017 gm. of ammonia, this, multiplied by the number of c. c. of acid neutralized, gives the quantity of ammonia in 25 c. c. of urine. The quantity in the twenty-four-hour specimen is calculated from this.

Determinations may be made in duplicate or triplicate by connecting two or more sets of apparatus in series.

(2) **The Vacuum Distillation Method.**—Shaffer[2] has modified the vacuum distillation method. As described by him, it is carried out in the following manner: To 50 c. c. of urine in flask *A* (Fig. 2) add an excess (15 or 20 gm.)

[1] The efficiency of the pump and its "working time" are readily tested by taking a specimen of urine and titrating the acid at the end of an hour; add more tenth normal acid and titrate at fifteen-minute intervals, until acid is no longer neutralized.

[2] Shaffer, P. "On the quantitative determination of ammonia in urine." *Amer. Jour. Physiol.*, 1903, VIII, 330.

of sodium chlorid, and about 50 c. c. methyl alcohol. In bottle *B* place 25 or 50 c. c. $\frac{N}{10}$ acid and in *B'* 10 c. c. $\frac{N}{10}$ acid, diluted in each case with a small amount of water. If too much water is added there will be danger of loss of acid by jumping over during the violent commotion which is set up in the acid by the rapid passage of the steam. If such a loss should occur the acid can always be recovered by rinsing out the filter flask *C*. When the apparatus is ready, about 1 gram dry sodium carbonate is added to the liquid in flask *A*, the stopper quickly put in place, and the suction started. With a good pump the pressure will be reduced to about 10 mm. Hg. in two or three minutes, when, the liquid surrounding *A* being at 50° C., a rapid boiling will begin. The temperature is maintained, and the boiling allowed to continue for fifteen minutes. At the end of that time the ammonia will in all cases have been completely given off, and the operation may be stopped by slowly letting in air at the stop-cock in tube *a*. The acid in *B* and *B'* is titrated and the ammonia calculated. One per cent. aqueous alizarin red is used as the indicator. (For a description of the end-point and the calculation see the preceding method of Folin.)

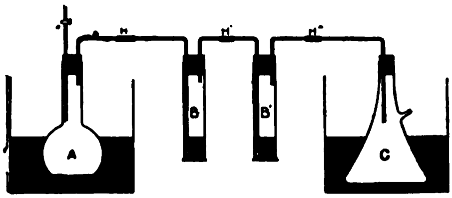


FIG. 2.—APPARATUS FOR THE DETERMINATION OF AMMONIA ACCORDING TO SHAFFER. (After Shaffer.)

This method is very accurate, and consumes very little time. Shaffer found the results in all cases correct within less than 10 mg. of ammonia per liter. Where the necessary apparatus is available, this method or that of Folin is to be preferred.

(3) **The Formalin Titration Method.**—This method was

first introduced by Ronchèse.[1] It is more accurate than the Schlösing method, and possesses an advantage over all other methods in the rapidity with which a determination may be completed. A number of observers have found it a satisfactory clinical method for following the ammonia excretion in acidosis, but, in its present form, it is not sufficiently accurate for metabolism experiments, since the results are apt to be too high, owing, in part at least, to the fact that aminoacids are determined with the ammonia. The principle of the method is based on an observation of Delépine, and is as follows: The addition of formalin to a solution of an ammonium salt gives rise to the formation of hexamethylenamin, with the liberation from the salt of the corresponding acid. If, then, the acid equivalents in the urine be first neutralized with alkali and formalin subsequently added, titration with tenth normal alkali will now reveal the acidity due to ammonium salts, and the quantity of alkali used indicates the amount of ammonia in the urine.

(a) *Method of Ronchèse.*[1]—Ten c. c. of the twenty-four-hour specimen of urine are measured with a pipette and placed in an Erlenmeyer flask of 300 c. c. capacity. To the urine add 100 c. c. of distilled water, previously boiled to drive off carbon dioxid, and then 1 or 2 drops of 0.5 per cent. alcoholic phenolphthalein solution. Under constant stirring add tenth normal sodium hydrate from a burette until a pale rose color makes its appearance throughout the fluid. Now add 20 c. c. of 20 per cent. formalin (commercial formalin is 40 per cent. strength), which has been neutralized, if necessary, with phenolphthalein as indicator, and again add tenth normal alkali, till the same color reaction

Ronchèse, A. "Nouveau procédé de dosage de l'ammoniaque." *Jour. de Pharm et de Chim.*, 1907, XXV (6th series), 611.

is obtained. This is the end-point. To the quantity of tenth normal alkali used after the addition of the formalin a correction of 0.1 c. c. is added for each 3 c. c. required in the titration. This sum equals the ammonia content of 10 c. c. of urine expressed in c. c. of tenth normal ammonia. One c. c. of tenth normal ammonia contains 0.0017 gm. ammonia. The quantity of ammonia in the twenty-four-hour specimen is calculated.

(b) *Method of Björn-Andersen and Lauritzen.*[1]—Twenty c. c. of urine, 5 drops of 0.5 per cent. alcoholic solution of phenolphthalein, and 20 gm. of finely powdered neutral potassium oxalate are placed in an Erlenmeyer flask, shaken vigorously about one minute, and then titrated immediately with tenth normal sodium hydrate under constant stirring, till a pale rose color is obtained. Now add 5 c. c. of commercial formalin (neutralized, if necessary), and the acid liberated will cause the color to disappear. Again titrate with tenth normal alkali to a pale rose color. (Add a little more formalin. If the color remains, the end-point has been reached; if it disappears, continue the titration until the color no longer fades on the further addition of a small quantity of formalin.) The quantity of alkali used to obtain the end-reaction after the addition of the formalin represents the number of c. c. of tenth normal ammonia in 20 c. c. of urine. Each c. c. of tenth normal ammonia contains 0.0017 gm. of ammonia.

The entire amount of tenth normal sodium hydrate used gives the "total acidity" of the urine. The authors find that the curves of total acidity and ammonia run parallel in health and in diabetic acidosis.

[1] Björn-Andersen, B., and Lauritzen, M. "Ueber Säure- und Ammoniakbestimmung im Harn und ihre klinische Anwendung." *Zeitschr. f. physiol. Chem.*, 1910, LXIV, 21.

The quantity of ammonia found is somewhat high in the presence of aminoacids.

(4) **Shaffer's Modification**[1] **of Schlösing's Method.**—This method, which requires at least two days for its completion, is more exact than Schlösing's, less accurate than Folin's, but for most purposes it meets the needs of the clinician. An advantage is the simple apparatus required. The ammonia, liberated under a bell jar or in a dessicator by the addition of stronger alkali, is neutralized by tenth normal acid. With a pipette, 25 c. c. of urine are placed in the bottom of a dessicator or dish having a diameter of 15 to 17 cm. An excess of sodium chlorid is added to prevent decomposition, then about 0.5 gm. of anhydrous sodium carbonate. A second smaller dish containing 20 c. c. of tenth normal sulphuric acid is placed in the dessicator or under a bell jar with the urine. It is essential that the dish containing the urine have a perfectly flat bottom and that the depth of the liquid be *not more than two mm.* "For the same amount of urine, *the wider the dish the more rapid will be the expulsion of the ammonia*" (Shaffer). The length of the operation may be reduced to forty-eight hours by letting the apparatus stand at 38° C. On longer standing at this temperature, the ammonia from decomposition becomes so great that the results are too high. At room temperature (about 25° C.) the apparatus is allowed to remain four days. The acid is then titrated with tenth normal sodium hydrate, using alizarin red (1 per cent. aqueous solution) as the indicator in the proportion of two drops to 200 to 300 c. c. of fluid, with a red color, not a violet, as the end-point. The ammonia in grams in 25 c. c. of urine is found by multiplying 0.0017 by the number of c. c. of $\frac{N}{10}$ acid neutralized.

[1] Shaffer, P. *Loc. cit.* (p. 15).

NITROGEN

The total nitrogen of the urine of a normal adult on a mixed diet lies usually between 10 and 16 gm., or about 0.2 gm. per kilo of body weight.

Kjeldahl's Method for Determination of Total Nitrogen.

Reagents: [1]

Crystalline copper sulphate.
Crystalline potassium sulphate.
Concentrated sulphuric acid.
40 per cent. solution of sodium hydrate.
Talc powder.
$\frac{N}{10}$ sulphuric acid; $\frac{N}{10}$ sodium hydrate.
One per cent. aqueous solution of alizarin red, or tincture of cochineal.

Method.—With a pipette 5 c.c. of the twenty-four-hour specimen of urine are measured into a Kjeldahl oxidizing flask (Jena glass) of about 800 c.c. capacity. Then add about 15 c.c. of concentrated sulphuric acid and about 0.2 gm. of copper sulphate crystals, and, finally, about 10 gm. of potassium sulphate. The flask is placed under a hood [2] and is heated over a Bunsen burner,[3] with a low flame at first, until the foaming has ceased. The heating is continued till the contents of the flask become clear. It may be

[1] To determine whether the reagents are N-free, proceed with the method, substituting 5 c. c. of glucose solution for the urine. If nitrogen is found, the necessary correction is evident.

[2] A lead pipe, perforated with holes to receive the necks of the digesting flasks (see Fig. 3) and connecting with a flue, is better than most hoods. If there is a good draught, the fumes are carried off perfectly. An outlet constructed of tile pipes is inexpensive and satisfactory.

[3] The most satisfactory form of apparatus is that designed by Folin and made by the International Instrument Co., Cambridge, Mass. The small model is shown in Fig. 3.

necessary to remove the flask, so that all the charred matter may be brought into the acid. After the fluid in the flask has become pale green or colorless, the heating is prolonged fifteen minutes to insure complete oxidation. All of the nitrogenous compounds have been converted to

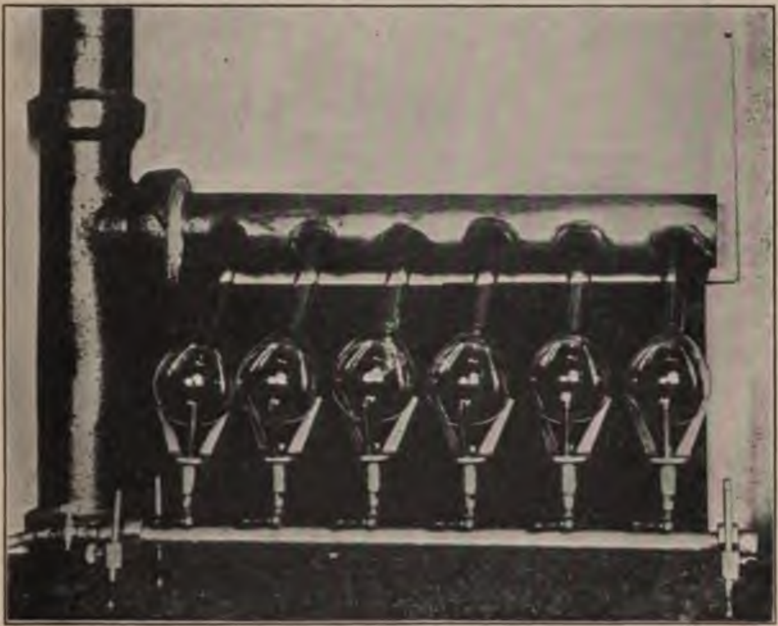


FIG. 3.—DIGESTING RACK FOR THE KJELDAHL NITROGEN DETERMINATION. The necks of the flasks extend into a perforated lead pipe, which is connected with a tile outlet.

ammonia, which unites with the sulphuric acid to form ammonium sulphate. The liquid is allowed to cool (it may crystallize eventually); about 300 c. c. of distilled water are then added, and, when solution is obtained, a heaping teaspoonful of talc powder is placed in the flask (to prevent bumping during the boiling). Finally, sufficient 40 per cent. sodium hydrate is added to render the solution

strongly alkaline; the quantity required must have been determined previously. It is well to incline the flask and pour the alkali down the side, to prevent mixing and possible loss of ammonia.[1] The flask is immediately connected with a distilling apparatus (Fig. 4) provided with a Hop-

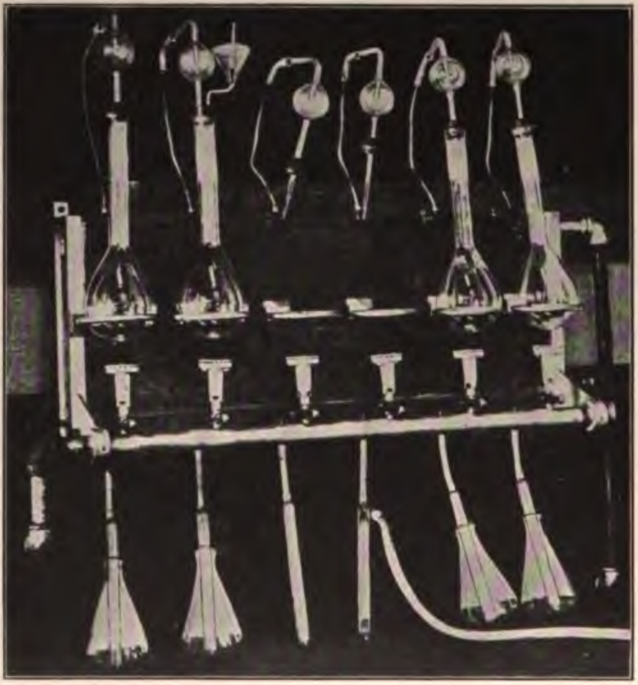


FIG. 4.—DISTILLING APPARATUS FOR THE KJELDAHL NITROGEN DETERMINATION.

kins bulb or similar device to prevent alkali passing over into the acid, and its contents boiled. The distillate, containing the ammonia liberated by the stronger alkali, is received in an Erlenmeyer flask in which 25 to 50 c. c. of tenth normal sulphuric acid have been placed. The distil-

[1] To guard against such loss, a doubly perforated stopper may be provided and the alkali introduced into the flask by a funnel, whose stem, passing through the stopper, is plugged immediately after adding the alkali.

lation is continued till the distillate is no longer alkaline to litmus—usually a half hour or less. The condensing tube is then washed with distilled water into the distillate. To determine the excess of acid, the contents of the flask are titrated with tenth normal alkali with alizarin red (2 drops to 200 to 300 c. c. of fluid) as the indicator. The end-point is a red color, not a violet. (Tincture of cochineal[1] is used at times as an indicator. It imparts a very pale brown color to acid solutions when added in the proportion of about four drops to 300 c. c.; when the reaction becomes alkaline the color changes to amethyst.) Since one c. c. of tenth normal acid is equivalent to 0.0014 gm. of nitrogen, the amount of the latter in 5 c. c. of urine and, finally, in the twenty-four-hour specimen is easily computed.

If insufficient acid has been taken to receive the distillate, the excess of ammonia may be titrated with tenth normal sulphuric acid (Hoppe-Seyler, Thierfelder).

Instead of distilling the oxidized material, the ammonia determination may be performed according to Folin's method. The titration and calculation of the result are performed as described above. Sodium hydrate is substituted for sodium carbonate.

CHLORIDS

In health, with the usual mixed diet, the chlorids of the urine usually amount to 10 to 15 gm. daily. The limits of the normal are said to be 6 and 22 gm.

Qualitative Test

About 10 c. c. of urine, placed in a test tube, are acidified with strong nitric acid, and then one or two drops of

[1] Tincture of cochineal is prepared by grinding cochineal bugs in 50 per cent. alcohol in a mortar, allowing the mixture to digest a day, and filtering.

dilute silver nitrate solution (10 to 15 per cent. aqueous solution) are added. A white precipitate denotes the presence of chlorids. An approximate idea of the quantity of chlorids may be gained. Normally, a dense precipitate appears and quickly settles to the bottom of the tube. With great reduction in the chlorids only a cloud is seen, without flocculent precipitate.

Quantitative Determination of Chlorids

Harvey's[1] Modification of Volhard's Method.—This method is valuable because of its rapidity without sacrifice of accuracy. As in the original method, the chlorids are precipitated by adding an excess of silver nitrate, the amount of which is determined by titration with ammonium thiocyanate. The chlorids are calculated as sodium chlorid. Albumin, if present in the urine in appreciable quantity, must be removed by boiling and the subsequent addition of dilute acetic acid, before proceeding to the estimation of the chlorids. Largely in the author's words, the method follows:

Reagents:

(a) A silver nitrate solution containing 29.042 gm. of chemically pure, crystalline silver nitrate in one liter of distilled water; 1 c.c. of this solution is equivalent to 0.01 gm. of sodium chlorid. (The silver solution may be standardized against a weighed quantity of dry, chemically pure sodium chlorid.)

(b) A solution of ammonium thiocyanate, 20 c.c. of which is equivalent to 10 c.c. of the silver nitrate solution. As this salt is very hygroscopic, it cannot be weighed with sufficient accuracy to make the solution directly. There-

[1] Harvey, S. C. "The quantitative determination of the chlorids in the urine." *Arch. Int. Med.*, 1910, VI, 12.

fore, 13 gm. of it are dissolved in one liter of distilled water, thus making a concentrated solution, whose strength is determined by titration against the silver nitrate solution, and the requisite dilution made. This is done in the following manner: 10 c. c. of the silver nitrate solution are measured with a pipette into a beaker, diluted with about 20 c. c. of distilled water, 2 c. c. of the indicator (sol. c) added, and the whole titrated with the ammonium thiocyanate solution. If, for example, 12 c. c. of the solution are used in the titration and the total volume of the thiocyanate solution is 960 c. c., the volume to which it must be diluted with distilled water is determined according to the equation 12:20::960:x, in which x represents the required volume.

(c) The *indicator* containing nitric acid. To 30 c. c. of distilled water add 70 c. c. of nitric acid (sp. gr. 1.2, or 33 per cent.). Saturate this menstruum with crystalline ferric ammonium sulphate and filter.

This indicator is recommended, inasmuch as it substitutes one solution in place of the two (the ferric indicator and the acid), and insures the use of the proper amount of the acid. Moreover, it is sufficiently concentrated, so that it is necessary to use only 2 c. c., and, therefore, it may be kept in a small reagent bottle. The stopper of this bottle may be a graduated dropper, which can at the same time serve to measure and transfer the indicator.

Method.—With a pipette transfer 5 c. c. of the twenty-four-hour specimen of urine (albumin-free) to a small beaker or Erlenmeyer flask, and dilute it with about 20 c. c. of distilled water.[1] The chlorids in this solution are now

[1] When the urine is highly colored, add 8 to 10 per cent. solution of potassium permanganate a drop at a time, until the red color no longer fades rapidly, and the urine has become pale yellow.

precipitated by adding 10 c. c. of the silver nitrate solution with a pipette. Next, place about 2 c. c. of the indicator in the mixture. The ammonium thiocyanate solution is then run in from a burette under constant stirring, until the first trace of red shows throughout the mixture. On allowing the precipitate to settle, the color may easily be recognized in the supernatant fluid. If, however, the mixture is stirred violently, the color will disappear. When the end-point appears on the addition of the first drop of ammonium thiocyanate solution (i. e., when the original 10 c. c. of silver solution is insufficient to precipitate all the chlorid), then 10 c. c. more of the silver nitrate solution are added, and the titration completed with corresponding allowance in the calculation.

The calculation may be made as follows: As 20 c. c. of the ammonium thiocyanate solution are equivalent to 10 c. c. of the silver nitrate solution, divide the number of c. c. of thiocyanate solution used by two (2) and subtract the quotient from 10 c. c., the amount of silver nitrate originally taken. The result is the number of c. c. of silver nitrate solution actually used in the precipitation of the chlorids. As 1 c. c. of the silver solution is equivalent to 0.01 gm. of sodium chlorid, the number of cubic centimeters of silver nitrate solution used, multiplied by 0.01, will give the amount of the chlorids, expressed in terms of sodium chlorid, in 5 c. c. of urine, the quantity taken. From this the total amount of chlorid in the twenty-four-hours specimen is calculated.

SULPHATES

Quantitative estimation of the sulphates in the urine is of no practical value in general clinical work at the present time. Of the sulphates present in the urine, indoxyl sulphate alone is tested for in the usual examination.

The tests for indoxyl sulphate depend on the oxidation of indoxyl to indigo blue and its extraction in chloroform. It is necessary at times to precipitate the urine, before testing, with one-fifth volume of 20 per cent. lead acetate to remove pigments, which may interfere with the recognition of the blue color.

Obermayer's Test.—Equal parts of urine and Obermayer's reagent (0.2 per cent. ferric chlorid in concentrated hydrochloric acid) are mixed in a test tube and allowed to stand a few minutes (2-3). A small amount of chloroform is added, and the test tube is inverted several times. With normal amounts of indoxyl sulphate a faint blue is seen in the chloroform; an excess causes a dark blue color. By using the same quantities of urine, reagent, and chloroform, and test tubes of uniform diameter, daily variations in the intensity of the reaction may be followed.

Jaffé's Test.—Equal quantities of urine and strong hydrochloric acid are mixed in a test tube; about 2 c. c. of chloroform and 1 to 3 drops of strong aqueous solution of calcium hypochlorite are added. The tube is inverted several times, and the indigo collects in the chloroform, as in the preceding test.

If the patient has been receiving *iodin* in any form, a violet color is imparted to the chloroform in performing Obermayer's and Jaffé's tests. To destroy the color produced by the iodin and bring out that of indigo blue, if present, the chloroform is transferred to a second test tube and is shaken with dilute potassium hydroxid; or water and a small quantity of strong sodium thiosulphate solution are added to the chloroform and the whole shaken. The violet is decolorized, leaving the blue.

Codein, when administered in large doses, is said to give a purplish red color to the chloroform.

ALBUMIN

Normal urine contains albumin in traces too small to be detected with the usual tests.

Before testing for albumin, two conditions must be fulfilled: (1) The urine must be perfectly clear, and (2) its reaction must be acid.

(1) If the specimen to be examined is fresh and fairly clear, passage through filter paper usually suffices to render it transparent and clear. With urines containing abundant fine precipitates or many bacteria, simple filtration is not satisfactory. Such urine should be shaken with Kieselguhr (infusorial earth) and then passed through a folded filter. The meshes of the paper are plugged, so that the filtrate is perfectly clear, though it may be necessary to return the first few cubic centimeters of the filtrate to the filter. Minute quantities of albumin may be removed by the filtration with Kieselguhr.

(2) If alkaline or neutral in reaction, the urine should be rendered slightly acid to litmus by the addition of a few drops of 3 per cent. acetic acid.

Of the following qualitative tests it is advisable to use at least two in all instances to avoid error. Heller's and the heat and acetic acid tests form a satisfactory combination.

Qualitative Tests

(1) **Heat and Acetic Acid Test.**—(a) First Method.—A test tube (18 to 20 mm. in diam.) is nearly filled with the clear, acid urine. Holding the tube by its lower end, the urine in the upper part is boiled over a Bunsen burner or spirit lamp, the cool urine in the lower part of the tube serving for comparison with the boiled portion. A cloud

may appear on boiling, due (1) to precipitation of calcium phosphate, or (2) to albumin, or (3) to the precipitation of both simultaneously. A few drops of 3 per cent. acetic acid are now added. If the precipitate be due to phosphates alone, it will disappear on the addition of the acid, whereas the albumin coagulum will usually be intensified, never lessened, unless a considerable excess of acid is added. When both phosphates and albumin are precipitated together, the cloud may be perceptibly diminished but not abolished by acidification. Very small quantities of albumin may give no cloud on heating, but the albumin may appear after the addition of the acid. Such traces of albumin are best detected by holding the tube against a dark background with the eye at a right angle to the source of light, for the faint cloud may be easily overlooked on casual inspection. The urine in the upper part of the tube (which has been boiled) is compared with the clear urine in the lower part of the test tube. When the urine is of very low specific gravity and, therefore, poor in salts, the test is improved by the addition of one-fifth to one-tenth volume of saturated sodium chlorid solution to the urine. The urine is not to be boiled after the addition of the acid.

The test is said to indicate albumin in a dilution of 1:130,000 (Glaesgen).

Sources of Error.—(a) There is danger in adding too much acetic acid, since the albumin may be converted into the soluble acid albumin or syntonin. That it requires a considerable excess of acid to redissolve the precipitate, however, once it is formed, is easily demonstrated. It is helpful to the worker to experiment with known specimens to determine the degree of latitude one can safely allow in the addition of the acid. (b) Nucleo[illegible] be precipitated by heat and acetic acid; it is also thrown out of

solution by the addition of dilute acetic acid to the *cold* urine. Two tests may be performed, one on the cold, the other on the boiled, urine; by comparison it is usually possible to estimate whether part or all of the precipitate is due to the nucleoproteid. Or, the urine is treated with dilute acetic acid, filtered to remove the precipitate of nucleoalbumin, a few more drops of the dilute acid added, and the contents of the test tube boiled; a precipitate appearing now is albumin. The test for nucleoalbumin is improved if the urine be diluted with water; that for albumin is sharper after the addition of salt. (c) Following the administration of cubebs, copaiba, turpentine, etc., resinous bodies appear in the urine, and may be precipitated. After cooling the fluid the precipitate may be dissolved in petroleum benzine or in alcohol, albumin being insoluble. (d) Albumoses appear after the urine becomes cool; the precipitate redissolves on boiling. (e) The Bence-Jones' body is coagulated at about 60° C., but usually redissolves in part or wholly as the boiling point is reached.

(b) SECOND METHOD.—This method, widely used in France, has recently been carefully examined and recommended by Glaesgen.[1] The acetic acid is added before the specimen is boiled. About 20 c. c. of urine and 5 drops of 20 per cent. acetic acid are mixed in a test tube. The urine in the upper part of the tube is boiled (or the mixture may be divided between two test tubes, one to be boiled, the other to serve as a control). If the acetic acid produces a cloud in the cold (nucleoproteid), the specimen is cleared by filtration before boiling. The acidification previous to boiling prevents a precipitation of phosphates in the major-

[1] Glaesgen. "Zur Methodik des Nachweises sehr kleiner pathologischer Eiweissmengen im Harn." *München. med. Wchnschr.*, 1911, LVIII, 1123.

ity of instances; if such a precipitate occurs, a few more drops of the acid are added to dissolve it. This will not cause the solution of a slight albuminous precipitate, provided the specimen is not reboiled. With the precautions given, the presence of a cloud or precipitate indicates albumin. (For the detection of a very faint cloud, see the first method.)

By this method Glaesgen finds that albumin may be demonstrated in a dilution of 1:180,000.[1]

(2) **Heat and Nitric Acid Test.**—The method of procedure is the same as in the preceding test (first method), the urine in the upper part of the test tube being boiled. One to four drops of concentrated nitric acid[2] are now added. The precipitate, which may form on boiling the urine, may be due to albumin or phosphates or to both. The phosphate precipitate is dissolved by the addition of the acid; in such case a few more drops of nitric acid are added, when albumin is precipitated, if present. When more than a trace of albumin is present in the urine, the precipitate is flocculent and whitish or brownish. With a urine of low specific gravity the addition of one-fifth volume of saturated sodium chlorid solution at times makes it possible to recognize a trace of albumin, which would otherwise be missed. The urine may remain clear after boiling, but a precipitate of albumin may still appear on acidification, as in the heat and acetic acid test. If the cloud is faint, there is danger of missing it, unless the tube be held against a dark background with the eye at a right angle to the source of light. Do not boil after adding the acid.

[1] A somewhat limited experience with the second method has shown it to be quite as sensitive as the first, in the writer's hands.

[2] Nitric acid becomes yellow from the formation in it of nitrous acid. It is readily cleared by the addition of crystals of urea.

The test is said to be as delicate as the heat and dilute acetic acid test.

Sources of Error.—The possibilities of error are much the same as in the heat and acetic acid test. (a) An excess of acid is to be avoided, as the precipitate may be dissolved, forming acid albumin. The proportion of acid to urine should not exceed about 1:1,000 (Simon). (b) Resinous bodies are distinguished as in the preceding test. (c) Uric acid may precipitate after standing a few minutes. The precipitate is crystalline, and gives the murexid test. (d) Albumose is soluble in the boiling solution, but insoluble in the cold. The precipitate which forms may be redissolved by heating. (e) Bence-Jones' protein usually exhibits maximal precipitation at about 60° C., with partial or complete disappearance of the coagulum at the boiling point. (f) In markedly icteric urine a green precipitate of biliverdin may be produced. This, unlike coagulated albumin, is soluble in alcohol. Finally, it may be added, the nitric acid possesses an advantage over dilute acetic acid, since its addition to boiling urine does not precipitate mucin or nucleoproteid. The nitric acid must be free from nitrous acid.[1]

(3) **Heller's Test.**—In performing this test a wide test tube or, better still, a conical glass or horismascope should be used. Ten to 20 c. c. of urine are placed in a conical glass, and then, with the glass inclined, concentrated nitric acid[2] is poured slowly down its side. Being the denser fluid, the acid collects at the bottom. The glass is now brought to the vertical position very gradually, to prevent mixing of the urine and acid. If albumin is present in the urine, a white precipitate is formed *at* the line of contact

[1] This[1] refers to footnote[2] on p. 31, beginning "Nitric Acid."
[2] See footnote on p. 31.

between urine and acid. The precipitate is acid albumin, which is insoluble in the great excess of acid. The breadth and sharpness of the ring will depend upon the quantity of albumin present, and also upon the success with which the urine and acid have been layered. When small quantities of albumin are present the ring may appear only after two or three minutes, and then may be overlooked unless the tube is examined against a dark background with the eye at a right angle to the source of light.

Glaesgen [1] finds the reaction positive with albumin in a dilution of 1:35,000.

Sources of Error.—(a) Urines which have been preserved with thymol may give a ring at the line of contact which is practically indistinguishable macroscopically from that produced by albumin.[2] Below the ring there is a greenish zone extending into the acid, above it a red zone. When thymol and albumin coexist, it may be noted that the thymol ring forms just beneath that of albumin. The thymol may be removed by shaking the urine with an equal volume of petroleum ether for two or three minutes. (b) Urates may be precipitated, but the ring is ½ to 1 cm. *above* the line of contact. The ring is broader than that caused by albumin, and disappears on warming the urine. (c) Nucleoalbumin may produce a ring ½ to 1 cm. *above* the line of contact. As nucleoalbumin is insoluble in strong acid, the ring rises as the acid diffuses upward in the urine. The ring is more marked if the urine be diluted with about three parts of water. (d) Resinous acids may form a ring above the line of contact. The ring is partially cleared on heating. The precipitate, if due to resins, may be pipetted off and dissolved in ether. When resinous bodies are sus-

[1] *Loc. cit.*

[2] Weinberger, W. "Thymol as a source of error in Heller's test for urinary protein." *Jour. A. M. A.*, 1909, LII, 1310.

pected the following test may be employed: To 8 to 10 c. c. of urine add 2 to 3 drops of strong hydrochloric acid; the resinous bodies are precipitated. Render strongly acid with hydrochlorid acid and heat; a red color develops. (e) Albumose and Bence-Jones' body form a ring at the line of contact, which disappears more or less completely on heating. (f) Urea nitrate may be deposited between the fluids. It is easily recognized, as it is not compact and uniform, but manifestly crystalline. Dilution of the urine causes its disappearance.

(4) **Potassium Ferrocyanide and Acetic Acid Test.**—To 10 to 15 c. c. of urine in a test tube add a few drops (about 5) of strong acetic acid to render the urine markedly acid. Nucleoalbumin, if present, is precipitated and should be removed by filtration. Now add a few drops of 5 per cent. potassium ferrocyanide. A cloud or a flocculent precipitate indicates albumin. Care must be exercised not to add an excess of the ferrocyanide, as the albuminous coagulum may be redissolved. The test is positive with albumin in a dilution of 1:70,000 (Glaesgen), but, like the preceding tests, its delicacy depends much on the concentration of the urine in salts.

Sources of Error.—Albumoses and Bence-Jones' protein are coagulated, but the coagulum disappears on heating—completely in the case of albumose, partially with Bence-Jones' body.

Numerous other tests for the recognition of albumin in the urine have been devised. Some of them, as Spiegler's, are too delicate. The tests given above have been thoroughly tested, and are almost universally employed by clinicians. Thorough familiarity with them should be sufficient for all practical purposes.

Quantitative Determination of Albumin

Tsuchiya's Modification of the Esbach Method.[1]—Tsuchiya has devised a new reagent for precipitating the coagulable protein, to be used with the Esbach tube. The formula of Tsuchiya's reagent is:

Phosphotungstic acid	1.5 gm.
Hydrochloric acid, conc...........	5.0 c. c.
Alcohol, 96 per cent., to...........	100.0 c. c.

Method.—If alkaline, the urine is acidified with a few drops of acetic acid to prevent bubbling, when the reagent is added. The Esbach tube is filled with urine to the mark U, and then the reagent is added to the mark R. The tube is corked and inverted twelve times to insure thorough and uniform mixing of the urine and reagent. (Do not shake, since bubbles clinging to the precipitate cause it to float.) The tube is placed in a vertical position for twenty-four hours at room temperature to allow the precipitate to settle, when the height of the precipitate is read on the scale marked on the tube. The figure obtained gives the quantity of albumin in grams *per liter.*

With large quantities of albumin the urine should be diluted with water, so that the reading will be below 4 gm. per liter, for Mattice has shown that above this the error increases greatly.

Tsuchiya's is a great improvement on the Esbach reagent, and should supplant it. With Esbach's reagent as the precipitant, the results are often not even approximately correct. Some of the advantages of Tsuchiya's reagent over that of Esbach are: (1) that the precipitate

[1] Mattice, A. F. "The quantitative estimation of albumin in the urine." *Arch., Int. Med.*, 1910, V, 313.

rarely floats, but (2) settles evenly in the bottom of the tube; (3) the readings are much less affected by slight variations in temperature; (4) the average error is very greatly reduced, amounting to less than 0.3 gm. per liter (controlled by the Kjeldahl and gravimetric methods), so that daily variations in albumin output can be followed with considerable accuracy, and (5) the reagent is clean, and does not stain hands or clothes (Mattice). Glucose in the urine does not interfere with the accuracy of the test.

Normal urines usually yield a slight precipitate when treated with Tsuchiya's reagent, but the bulk of it is so small that it is not measurable, and in no way interferes with the test.

Removal of Albumin from the Urine.—As albumin interferes with certain reactions, it is necessary at times to remove it before performing other tests. A convenient method is the heat and dilute acetic acid test. The coagulated protein is removed by filtration, and the filtrate tested by one of the other tests to determine whether it is albumin-free.

BENCE-JONES' BODY

This protein is of rare occurrence. There is no simple, decisive, qualitative test by which it may be recognized. Its presence may be strongly suspected, though not absolutely proved, by the following reactions: If alkaline or neutral, acidify the urine with dilute acetic acid; filter the specimen, if necessary, to render it clear. (1) On heating the urine slowly in a test tube there appears a milky turbidity at about 52° C.; at 60° C. the precipitate is abundant and sticky. After the temperature rises above 70° C.

the precipitate usually lessens materially, and may entirely disappear at the boiling point, though a slight cloud usually persists. As the urine cools the precipitate reappears. (2) The addition of an excess of nitric acid to the cold urine causes a precipitate, which is partially or completely dissolved on boiling, but again separates as the temperature becomes lower. (3) Similar reactions may be obtained with many of the tests for albumin. That all of these reactions are influenced very greatly by the acidity and the salt content of the urine has been shown by Massini.[1] The further identification of Bence-Jones' protein is more or less complicated; the reader is referred to the larger works or to the literature.

ALBUMOSE

The secondary or deuteroalbumoses, though of wide occurrence in the urine in disease, are ordinarily of little diagnostic importance. Their presence may be shown in the following manner (Simon): Strongly acidify a few c. c. of urine with acetic acid, and then add an equal volume of saturated solution of sodium chlorid. The presence of albumose is indicated by the occurrence of a precipitate, which disappears on boiling and reappears on cooling. Since albumin is usually present in the urine with albumose, the boiling urine should be filtered to remove the albuminous precipitate. A cloud, which develops in the filtrate on cooling, signifies albumose. To the hot filtrate an excess of sodium hydrate is added to render it strongly alkaline, then 1 per cent. copper sulphate drop by drop, when a red color appears (the biuret test).

[1] Massini, R. "Untersuchungen bei einem Falle von Bence-Jones'scher Krankheit." *Deutsch. Arch. f. klin. Med.*, 1911, CIV, 29.

GLUCOSE

(Dextrose, Grape Sugar)

Glucose is present normally in the urine in traces, the quantity varying between 0.015 and 0.04 per cent. in the twenty-four-hour specimen. The amount is so small that it is not detected with the usual clinical tests.

The urine to be tested should be clear. Simple filtration may be sufficient. If this fails the urine is shaken with powdered normal lead acetate, and then filtered.

Qualitative Tests

(1) **Trommer's Test.**—If more than a trace of albumin is present, it should be removed with heat and dilute acetic acid. To urine in a test tube add one-third volume of 10 per cent. sodium or potassium hydrate, then 10 per cent. copper sulphate solution—the contents of the tube being thoroughly mixed after each addition of the copper—until a slight excess of cupric hydroxid remains undissolved. If sugar is present in the urine, much more copper sulphate can be added before a permanent precipitate is obtained, and the percentage of sugar may be roughly estimated in this way; the mixture turns deep blue. The upper part of the fluid is now heated just to boiling. In the presence of glucose cuprous oxid and hydroxid are formed, producing in the heated portion greenish-yellow clouds, which gradually change to a brick red and diffuse throughout the fluid. The rapidity and intensity of the reaction depend upon the concentration of the glucose. With a high percentage of sugar, metallic copper may separate as a brownish-red coating on the side of the test tube (easily removed with nitric acid).

The test will indicate 0.2 per cent. of dextrose. It should always be confirmed by other tests.

Sources of Error.—When the reduction of the copper is atypical, the interpretation of the result is in doubt. The *combined glycuronates, uric acid, creatinin, creatin,* are all capable of reducing copper to a certain extent. They never cause more than a dirty yellow; the granular, red precipitate of cuprous oxid is missed, for ammonia, creatinin, etc., keep in solution the small amounts of cuprous oxid formed in sugar-free urines. With *less than* 0.2 *per cent. of glucose,* a similar result may be obtained, for the sugar itself may hold in solution a small quantity of cuprous oxid; on cooling the red, granular precipitate may appear. The *alkaptone bodies* may also cause an atypical reduction. *Other hexoses* or *pentose* may be responsible for the reaction. Before testing with any copper solution, *chloroform* must be removed from the urine by boiling, as it is a fairly strong reducing agent. Urine preserved with *formaldehyde* may likewise give a reduction.

(2) **Fehling's Test.**

Solution (1):[1]

Copper sulphate, cryst..........	34.65	gm.
Distilled water to...............	1,000.0	c. c.

Solution (2):[1]

Rochelle salt	173.0	gm.
Sodium hydrate	125.0	gm.
Distilled water to...............	1,000.0	c. c.

More than a trace of albumin should be removed from the urine before testing. Equal volumes of solutions (1)

[1] If solution (1) is to be used for *qualitative work only*, it is not necessary to weigh the copper exactly on an analytical balance. In preparing solution (2), dissolve the Rochelle salt in hot water, then cool to room temperature, add the sodium hydrate and make up to one liter.

and (2) are mixed [1] in a test tube and boiled; the deep blue fluid should remain perfectly clear. Now (a) add the urine in small amount, never exceeding one-half the volume of the mixed solutions originally taken. A yellow or red precipitate appears at once. A second way (b) of performing the test is to layer the urine over the mixed, boiled solutions by allowing it to run down the side of the test tube. At the line of contact a yellow precipitate, which quickly turns red and diffuses downward, is formed in the presence of glucose. The precipitate appears within a few seconds. With small amounts of glucose, the diffusion downward is lost, but the red oxid soon collects at the bottom of the tube. With the second procedure (b) there is less likelihood of confusion in interpreting the test.

The test is said to reveal 0.08 per cent. of glucose.

Sources of Error.—A dirty, greenish-yellow precipitate does not mean sugar in the majority of instances. The test contains all the sources of error of Trommer's test (q. v.). Chloroform, when used to preserve the urine, must be driven off by boiling. A precipitate which appears on standing means nothing.

(3) **Almén-Nylander's Test.**—Reagent. Four grams of Rochelle salt are dissolved in 100 c. c. of warm 10 per cent. sodium hydrate. The mixture is saturated with bismuth subnitrate (add about 2.0 gm. of the latter), filtered, and placed in a dark bottle. The reagent is permanent.

Albumin must be removed from the urine, since the sulphid of bismuth, which may result from its presence, is brown and interferes with the test.

To the urine in a test tube add one-tenth volume of the reagent, mix, and place the tube in a boiling water bath

[1] A mixture of the two solutions is not permanent, and should, therefore, always be *freshly prepared* at the time of performing the test.

for *five minutes.*[1] More prolonged boiling should be avoided, otherwise sugar-free urine may reduce the bismuth. If dextrose is present the fluid darkens, and a black precipitate of metallic bismuth separates. When the solution turns dark only on cooling the test is negative. In a sugar-free urine a white precipitate of phosphate is formed.

The test indicates 0.08 per cent. of glucose, maltose, or lactose, and 0.07 per cent. of levulose (Rehfuss and Hawk).

Sources of Error.—Nylander's solution is *not* reduced by uric acid, creatinin, the alkaptone bodies, pyrocatechin, and phosphates, and the test is, therefore, a good control of Trommer's and Fehling's tests. *Pentose* may cause a reduction; the same is true of *hexoses.* The test may be positive after eating *asparagus,* and also after the administration of *hexamethylenamin* (urotropin). An excess of *combined glycuronates* may cause a reduction. *Chloroform* should be removed from the urine by boiling. *Formaldehyde,* when added to the urine, reduces the bismuth.

Rehfuss and Hawk agree with Kistermann that any protein-free urine which gives a negative Nylander's test may safely be said to be sugar-free in a clinical sense. It is safer than either of the copper tests, and should be used more extensively than it is.

(4) **The Fermentation Test.**—When positive, this test proves that the reducing body is a fermentable sugar. In the vast majority of instances the sugar is glucose.

A piece of fresh compressed yeast about the size of a hazel nut is rubbed in a mortar with about 50 c. c. of urine, which is then filled into a fermentation tube, so that the air is completely displaced. As controls, use (a) normal urine and yeast, and (b) normal urine and yeast plus glu-

[1] Rehfuss, M. E., and Hawk, P. B. "A study of Nylander's reaction." *Jour. Biol. Chem.*, 1909-10, VII, 273.

cose, to prove the activity of the yeast. The three tubes are set aside in a warm place (temperature 20° to 37° C.) for several hours. If the yeast is active and glucose present, alcohol and carbon dioxid gas will be evolved, the bubbles collecting at the top of the tube. No gas, or only a minute bubble, should be evolved in the control tube (a), whereas the glucose added to control tube (b) should be fermented. To lessen the danger of bacterial decomposition, the urine may be boiled before testing. The test will indicate 0.05 to 0.1 per cent. of glucose. As a further check the reduction tests may be repeated with the filtered urine after fermentation is completed.

A positive test indicates the presence of a fermentable sugar.

Sources of Error.—Levulose and *maltose,* if present, may be fermented with the evolution of gas. Before adding the yeast *chloroform* must be removed from the urine by boiling. *Thymol* and *formaldehyde,* when used as preservatives, may inhibit the growth of the yeast. It is said that *hexamethylenamin* in sufficient doses also prevents the fermentation.

(5) **Cippolina's[1] Modification of the Phenylhydrazin Test.**—Albumin, when present, should be removed before performing the test.

To 4 c. c. of urine in a test tube are added 5 drops of pure phenylhydrazin (the base) and 0.5 c. c. of glacial acetic acid (or 1.0 c. c. of 50 per cent. acetic acid); the mixture is boiled gently over a low flame for one minute. Now add 4 to 5 drops of sodium hydrate (sp. gr. 1.160); the mixture must still remain acid. The whole is heated a few seconds longer, and set aside to cool. Immediately or within

[1] Cippolina, A. "Ueber den Nachweis von Zucker im Harn." *Deutsche med. Wchnschr.,* 1901, XXVII, 334.

about twenty minutes, especially with a urine of low specific gravity, the characteristic sheaf-like yellow needles of phenylglucosazone appear. Since their size is subject to considerable variation, high magnification is necessary at times to see them.

The test is very delicate, indicating 0.05 per cent. of glucose. However, the sensitiveness of the test depends very largely upon the specific gravity of the urine. Concentrated urines may react negatively in the presence of less than 0.2 per cent. of glucose. The reason for this is that phenylglucosazone crystals are held in solution in the presence of much urea, ammonium salts, and other nitrogenous bodies. But with more than 0.2 per cent. of glucose typical crystals form within a few minutes, regardless of the specific gravity of the urine.

In place of the characteristic needles yellow balls, which change to thorn-apple forms or rosettes, may be obtained. The latter are seen only in urine containing a pathological quantity of sugar, never in a normal urine (Cippolina). Characteristic needles arranged in sheaves may be obtained by recrystallization from hot 60 per cent. alcohol. When the crystals are atypical the specimen should be set aside and reëxamined at the end of one hour.

To determine definitely that the crystals are derived from glucose and not from another sugar, it is necessary to filter them off and purify them by repeated recrystallization from hot 60 per cent. alcohol. (The melting point of the purified crystals may be determined [1]; that of phenylglucosazone is 204 to 205° C. The melting point of levulosazone is the same, while maltosazone crystals melt at

[1] For a description of methods, with critical discussion, see Menge, G. A. "A study of melting-point determinations." *Bull. No. 70*, Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1910.

about 207° C. The value of melting point determinations for the identification of one of the three sugars mentioned is, therefore, not great, though very helpful in differentiating the osazone of pentose, melting point 168° C., somewhat less so with lactose, 200° C.)

The dry, purified crystals may be identified by dissolving 0.2 gm. of them in 4 c. c. of pure pyridin, to which 6 c. c. of absolute alcohol are subsequently added, and the whole well mixed. The 100 mm. tube of the polariscope is then filled with this mixture. Phenylglucosazone gives a *levorotation* of—1° 30′. This procedure is seldom, if ever, necessary in clinical work.

Quantitative Estimation of Glucose

(1) **Benedict's [1] First Method.**—This is one of the best and quickest quantitative methods for the clinician. The solutions required are:

Solution A:

Recrystallized copper sulphate [2]..	69.3 gm.
Distilled water to...............	1,000.0 c. c.

Solution B:

Crystalline Rochelle salt.........	346.0 gm.
Anhydrous sodium carbonate....	200.0 gm.
Distilled water to...............	1,000.0 c. c.

Solution C:

Potassium sulphocyanide	200.0 gm.
Distilled water to...............	1,000.0 c. c.

[1] Benedict, S. R. "The detection and estimation of reducing sugars." *Jour. Biol. Chem.*, 1907, III, 101; also *N. Y. Med. Jour.*, 1907, LXXXVI, 497.

[2] This must be accurately weighed on an analytical balance. Sols. B and C do not require exact weights of the substances.

For use, these solutions are mixed in equal proportions *in the order in which they are given.* As the mixed solution (which is blue) keeps fairly well, it is practicable to prepare 300 c. c. or more, according to the demand. For measuring, pipettes or standard flasks are required.

Thirty c. c. of the mixed solution are transferred with a pipette to an evaporating dish. To this are added 2.5 to 5.0 gm. of anhydrous sodium carbonate, in order to increase the alkalinity of the fluid. (For titrating dilute sugar solutions, the larger quantity of carbonate will be required, since the greater amount of urine which must be added will diminish the concentration of the alkaline salt.) The mixture is now placed over a Bunsen burner and is boiled, until the carbonate is dissolved. A small piece of washed absorbent cotton is added to prevent bumping. Urine is now run into the boiling solution from a burette until a heavy chalk-white precipitate of cuprous sulphocyanide is formed, and the blue color of the fluid begins to lessen perceptibly. The remaining portions of urine should be added in quantities of from two to ten drops (depending on the depth of color remaining and the relative strength of the sugar solution), with vigorous boiling of about one minute between each addition. The end-point is the complete disappearance of the blue color. The point is very sharp, and may be obtained with a single drop. If the precipitate be allowed to settle, the color in the supernatant fluid is more easily appreciated.

For the complete reduction of the copper contained in 30 c. c. of the mixed solution, 0.073 gm. of glucose are required. The quantity of urine used from the burette, therefore, contains 0.073 gm. of glucose. From this value the amount of glucose in the twenty-four-hour quantity of urine is calculated.

When the urine is highly colored, its addition to the mixed solution may leave a yellowish supernatant fluid. To avoid this the urine may be decolorized by first shaking it with finely powdered normal lead acetate. The filtered urine is then almost colorless.

Urine which has been preserved with chloroform may cause a precipitate of the red oxid to form in place of the white cuprous sulphocyanide. This difficulty is obviated by first boiling the urine to drive off the chloroform. It may also be overcome by substituting for solution C the following:

Solution D:

Potassium ferrocyanid	30.0	gm.
Potassium sulphocyanid	125.0	gm.
Anhydrous sodium carbonate.....	100.0	gm.
Distilled water to...............	1,000.0	c. c.

(2) **Benedict's**[1] **Second Method.**—This method appears to be an improvement on Benedict's first method, in that the three solutions are replaced by one, which is permanent. As in the preceding method, a white precipitate of cuprous sulphocyanid is formed. Benedict's directions for the preparation of the solution and for the titration follow:

Crystallized copper sulphate.....	18.0	gm.
Anhydrous sodium carbonate[2]...	100.0	gm.
Sodium citrate	200.0	gm.
Potassium sulphocyanate	125.0	gm.
Five per cent. potassium ferrocyanid solution	5.0	c. c.
Distilled water to...............	1,000.0	c. c.

[1] Benedict, S. R. "A method for the estimation of reducing sugars." *Jour. Biol. Chem.*, 1911, IX, 57.

[2] 200.0 gm. of the crystallized salt may be used.

With the aid of heat dissolve the citrate, carbonate, and sulphocyanate in enough water to make about 800 c. c. of the mixture, and filter. Dissolve the copper sulphate separately in about 100 c. c. of water, and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanid solution cool, and dilute to exactly one liter. Of the various constituents, *only the copper sulphate need be weighed with exactness.* Twenty-five c. c. of the reagent are reduced by 0.050 gm. of glucose, or by 0.053 gm. of levulose.

Method.—With a pipette measure 25 c. c. of the reagent into a porcelain evaporating dish (25 to 30 cm. in diameter) and add 5 to 10 gm. of anhydrous sodium carbonate (or twice the weight of the crystallized salt), and a *very small quantity* of powdered pumice stone. Heat the mixture to vigorous boiling over a free flame till the carbonate is dissolved, and from a burette run in the twenty-four-hour specimen of urine (diluted accurately 1:10, unless the sugar content is known to be very slight) quite rapidly, until a heavy white precipitate is produced, and the blue color of the solution begins to diminish perceptibly. From this point the urine is run in more and more slowly, with constant vigorous boiling, until the disappearance of the last trace of blue color, which marks the end-point. An interval of 30 seconds' vigorous boiling should be allowed between each addition of urine.

The following explanatory points may be added regarding the solution: When ready mixed, the solution appears to keep indefinitely without any special precaution, such as exclusion of light, etc. The trace of ferrocyanid is added to prevent precipitation of red cuprous oxid, which may be caused by certain impurities. *Chloroform has such a marked tendency in this respect that it must not be pres-*

ent during the titration. The additional alkali is added prior to the titration in order to provide sufficient alkalinity to insure a sharp end-point. Should the mixture become too concentrated during the titration process, distilled water may be added to replace the volume lost by evaporation.

(3) **Polariscopic Determination.**—The polariscope is an expensive instrument, and for this reason it is not as generally employed for sugar determinations as the rapidity and ease of its use would seem to warrant. For clinical use the instrument is supplied with two specially made tubes, 94.7 mm. and 189.4 mm. long, which permit a direct percentage reading of glucose; the short tube is used with dark, highly colored urines, the readings obtained being divided by two. The tubes must be perfectly clean and dry before using; hot water or fluid should not come in contact with them, since the expansion of the glass against the outer brass tubing may crack the former.

The twenty-four-hour specimen, *acid* in reaction, is filtered and decolorized, if necessary. This is best accomplished by the addition of about 2 gm. of finely powdered normal lead acetate to the urine, which is then shaken vigorously and filtered. The first cloudy portions of the filtrate are returned to the filter, until the filtrate, which is almost colorless, is perfectly clear. Practically no sugar is held back by the normal lead acetate.[1] The clear urine is now filled into the polariscope tube (189.4 mm. in length) until the fluid is convex above the end of the tube. The glass disc is then placed over the end of the tube and secured in place by screwing down the metal cap. Air bubbles

[1] [illegible] "Ueber Klärung und Entfärbung." *Biochem. Ztschr.*, [illegible] XXIV, [illegible]

must be avoided, since their presence makes a satisfactory reading impossible. The tube is now placed in the polariscope, which must be in a dark room. For illumination a sodium flame is used. After focusing, readings are made, first without the urine, to determine whether the zero point is accurate, next, after refocusing, with the tube of urine; starting at zero, the handle is rotated until the entire field is equally illuminated. At least six readings should be made. The percentage is read directly from the scale, tenths being obtained on the vernier. (In case the instrument is supplied only with the standard tubes of 100 and 200 mm. length, the percentage of glucose may be calculated from the polariscopic readings by dividing the results by 0.527.)

The method gives fairly satisfactory results. When no disturbing bodies are present in the urine, the error is about 0.1 per cent. of glucose.

Sources of Error.—(1) *Albumin,* when present, must be removed before making polariscopic determination of glucose, otherwise the albumin, which is levorotatory, will counterbalance the dextrorotatory glucose, in part at least. (2) *Alkalinity* of the urine precludes its use with the polariscope, since it has been shown that in alkaline media dextrose may be converted into levulose.[1] The addition of a preservative to the specimen usually suffices to prevent an acid urine becoming alkaline. (3) *β-oxybutyric acid* is levorotatory, and its presence, therefore, interferes with the accurate estimation of glucose. (4) The *combined glycuronates* are levorotatory, though they are generally present in such small quantity as to produce only slight rotation of the polarized light. (5) *Levulose,* when present

[1] Koenigsfeld, H. "Zur Klinik und Pathogenese der Lävulosurie beim Diabetes mellitus." *Ztschr. f. klin. Med.,* 1910, LXIX, 291.

in the urine with glucose, is antagonistic, and lowers the reading for glucose. (6) *Maltose* is occasionally present in the urine with glucose. Since it is more powerfully dextro-rotatory than glucose, the reading may give a value which is too high.

From the foregoing it is apparent that the error arising from β-oxybutyric acid may be estimated approximately by making polariscopic examination of the specimen before and after fermentation with yeast. With the combined presence of glucose and levulose, the relative proportions of each may be determined with a fair degree of accuracy by comparison of the value obtained by titration with copper solution and the polariscopic value.

(4) **Robert's Specific Gravity Method.**[1]—This method depends upon a lowering of the specific gravity of the urine as a result of fermentation of the sugar. By obtaining the specific gravity of the fermented and the unfermented urine, the quantity of sugar may be calculated. About 2.0 gm. of compressed yeast are rubbed in a mortar with 50 c. c. of the twenty-four-hour specimen of urine, acidified with acetic acid if necessary. The specific gravity of the suspension is taken at once, the temperature of the mixture being noted. The mixture is set aside in a warm temperature (25 to 37° C.) in a receptacle plugged with cotton, or, better, in a large fermentation tube. When fermentation is complete the yeast settles to the bottom of the flask; it is well, nevertheless, to test the fluid to determine the complete disappearance of the sugar. The mixture is well stirred and a small portion removed. It is filtered and tested with Fehling's solution. If glucose still remains, the fermentation is allowed to continue, until there

[1] Lohnstein, Th. "Ueber die densimetrische Bestimmung des Traubenzuckers im Harne." *Arch. f. d. ges. Physiol.*, 1895-6, LXII, 82.

is no longer a reduction of the copper. The mixture is again stirred thoroughly to restore the suspension, and the specific gravity is determined the second time. It is important that the temperature of the suspension at the times of determining specific gravity does not differ by more than 1° C. The usual urinometers are too inaccurate for the determination of the specific gravity, which should be carried to the fourth decimal place. Lohnstein's instrument (Fig. 5) is convenient and satisfactory for the purpose. It is an areometer, in whose stem the pan, A, is mounted. It is floated in the urine and weights are placed on the pan until the shelf, C, is exactly on a level with the surface of the fluid. The sum of the weights on the pan is the specific gravity of the fluid. If the pan is loaded too heavily, so that the surface, C, sinks into the fluid, it must be removed and dried.

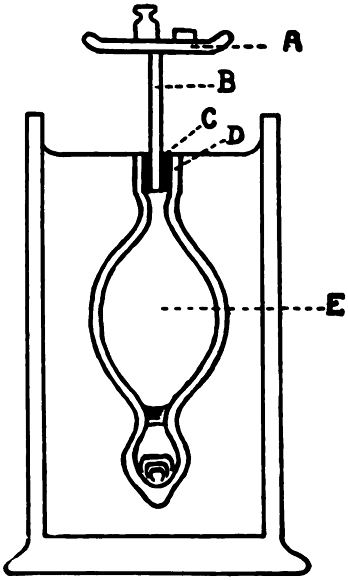


FIG. 5.—LOHNSTEIN'S AREOMETER. A, pan for weights; C, shelf which should be level with the surface of the fluid; E, air chamber. (After Lohnstein.)

The quantity of sugar is calculated by multiplying the difference in the specific gravities by the factor 234. The result is glucose in grams per cent. When unfiltered urine is used (i. e., for the second determination, after fermentation is completed), the error does not exceed 5 per cent. (Lohnstein). The method permits the determination of glucose in strengths of 0.1 per cent. or more.

(5) **Measurement of the carbon dioxid gas formed dur-**

ing fermentation has been used to determine the dextrose content of urine. One of the most widely known forms of apparatus for this purpose is Einhorn's. More accurate results have been obtained with Lohnstein's apparatus (Fig. 6). This consists of a J-shaped tube mounted on a stand. In the short arm of the tube a bulb is blown, the outlet of which may be closed by a glass stopper. A hole in the neck may be brought opposite a similar opening in the hollow stopper. The long arm is provided with a scale. A quantity of mercury and a pipette for measuring the urine are supplied with the apparatus.

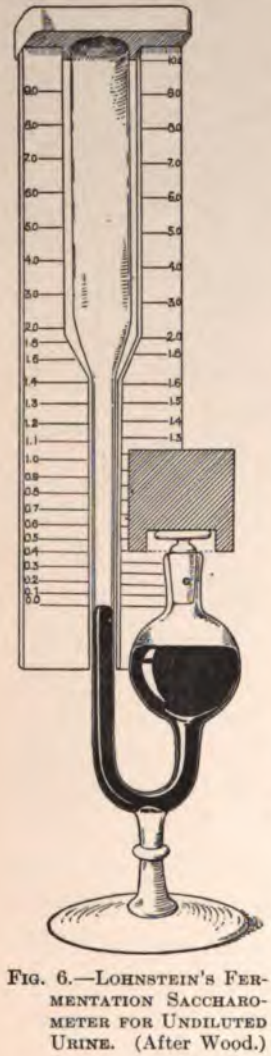


FIG. 6.—LOHNSTEIN'S FERMENTATION SACCHAROMETER FOR UNDILUTED URINE. (After Wood.)

METHOD.[1]—The mercury is poured into the apparatus. The bulb is partly filled, and mercury extends a short distance up the long arm of the tube. Then, with the pipette, 0.5 c. c. of urine and 0.1 to 0.2 c. c. of a yeast suspension[2] (1 part of compressed yeast to 2 to 3 volumes of water) are placed in the bulb on the surface of the mercury. The glass stopper (greased with vaselin 20 per cent., in yellow wax) is placed in the neck of

[1] Lohnstein, T. "Ueber Gärungs-Saccharometer nebst Beschreibung eines neuen Gärungs-Saccharometers für unverdünnte Urine." *München. med. Wchnschr.*, 1899, XLVI, 1671.

[2] The quantity of yeast suspension employed depends upon the concentration of glucose. With very low percentage of sugar the yeast may be rubbed up with 10 to 15 volumes of water.

the bulb in such a way that the openings are opposite one another; this is to avoid a positive pressure on inserting the stopper. The scale is now placed on the long arm of the tube; the zero line should correspond with the level of the mercury in the long arm. The stopper is turned, to close the bulb, and a weight (provided with the apparatus) is placed over it to prevent it from being blown out, as the carbon dioxid forms. The apparatus is placed in an incubator at a temperature of 32 to 38° C. for 4 to 5 hours, or at room temperature for 24 hours. The carbon dioxid formed in the bulb from fermentation of the sugar forces the mercury into the long arm of the tube. The percentage of glucose is read directly from the scale, which is provided with two columns of figures, one for the average room temperature, the other for body heat.

LEVULOSE

Levulose, when present in the urine, is usually associated with dextrose. Occasionally it is the only sugar in the urine, a few cases of levulosuria having been reported.[1] Levulose responds to most of the tests for glucose. It is fermentable, reduces copper and bismuth, gives the phenylhydrazin test; there are, however, certain dissimilarities by which the two sugars may be separated.

(1) **Seliwanoff's Test, as Modified by Borchardt.**[2]—Five to 10 c. c. of urine and an equal volume of 25 per cent. hydrochloric acid (i. e., 2 parts of concentrated hydrochloric acid and one part of water) are mixed, and a few grains

[1] Strouse, S., and Friedman, J. C. "Lævulosuria." *Arch. Int. Med.,* 1912, IX, 99.

[2] Borchardt, L. "Ueber die diabetische Lävulosurie und den qualitativen Nachweis der Lävulose im Harn." *Ztschr. f. physiol. Chem.,* 1908, LV, 241.

of resorcin added. The mixture is boiled gently for a few seconds. A red color appears, usually followed by a brownish precipitate, if levulose is present. The fluid is now cooled, poured into an evaporating dish or beaker, and treated with sodium carbonate in substance until the reaction of the mixture becomes alkaline. It is then returned to a test tube, and shaken with acetic ether (ethyl acetate). In the presence of levulose the acetic ether is colored yellow. The test indicates 0.05 per cent. of levulose.

Sources of Error.—(1) The simultaneous presence of nitrites and indican in considerable quantity may yield a positive reaction. The nitrites may be destroyed by acidifying the urine with acetic acid and boiling for one minute. (2) Large quantities of indican alone may interfere with the reaction by imparting a blue color to the acetic ether, making it impossible to recognize the yellow color from levulose. In such case the indican is removed by treating the urine with an equal volume of Obermayer's reagent and extracting several times with chloroform, until the latter is no longer colored blue. The fluid is then poured into a fresh test tube, the chloroform being discarded, and is diluted with one-third volume of water, in order to reduce the strength of the hydrochloric acid to 12 to 13 per cent. A knife point of resorcin is now added, and the Seliwanoff test carried out. (3) Urorosein, when abundant in the urine, may impart a reddish-violet color to the acetic ether. To remove the pigment before applying Seliwanoff's test, take equal quantities of urine and 25 per cent. hydrochloric acid, and extract the mixture two to three times in a separating funnel with amyl alcohol. Discard the amyl alcohol, which contains the urorosein, add resorcin, and proceed with the test in the usual way. (4) It has been found

that patients taking santonin or rhubarb may give a positive Seliwanoff reaction. Discontinuance of the drug causes the reaction to disappear.

In performing the test prolonged boiling should be avoided. Borchardt finds no interference with the reaction from the presence of glucose, lactose, maltose, arabinose, or glycuronic acid compounds. Saccharose may yield a positive reaction, since boiling it with acid liberates levulose.

(2) **The phenylhydrazin test** (see p. 42) gives crystals of phenyllevulosazone. They are indistinguishable microscopically from phenylglucosazone; the melting point of each is the same. The crystals can be positively identified by determining their rotation of polarized light. They are purified by repeated crystallization from hot 60 per cent. alcohol. Then 0.2 gm. of the pure crystals are dissolved in 4 c. c. of pyridin, and 6 c. c. of absolute alcohol are added. The mixture is poured into the 100-mm. tube of the polariscope and examined. Levulosazone gives a dextrorotation of 1° 20′.

Levulosuria combined with glycosuria should be suspected when the quantity of glucose found on polariscopic examination falls short of that shown by titration. A positive Seliwanoff reaction and the lack of a levorotatory body after fermentation practically confirm it.

Pure levulosuria offers no difficulties in recognition, if access to a polariscope may be had. The levorotation of the urine, together with positive Seliwanoff and reduction tests and the presence of a fermentable substance, makes the identification sufficiently complete, if all tests are negative after fermentation. The phenylhydrazin test, as described above, removes all doubt, if positive. Levulosuria

is usually unsuspected, unless the urine be examined with a polariscope.

Alimentary levulosuria has been used in hepatic diagnosis. In the absence of derangement of hepatic function an individual can take 100 gm. of levulose on a fasting stomach without the subsequent appearance of levulose, as a general rule. On the other hand, the majority of patients with liver disease exhibit levulosuria under such conditions.[1]

MALTOSE

Maltose is occasionally present in the urine, usually in association with glucose. It reduces copper and bismuth solutions, and is fermentable. Maltose has about two and one-half times the dextrorotatory power of glucose, whereas its reducing power is only about two-thirds that of glucose. Therefore, its presence may be suspected when polariscopic values exceed the results found with the reduction methods.

LACTOSE

Lactose may appear in the urine of women physiologically in connection with stasis of milk in the breasts. Cupric salts are reduced more slowly than by glucose. Ammoniacal silver nitrate is reduced by lactose in the cold. Lactose is not fermentable by yeast. If equivocal results are obtained with the usual compressed yeast, the fermentation may have been due to contaminating bacteria. With a pure culture of Saccharomyces apiculatus, lactose is not fermented.

[1] For a discussion of this test, see Churchman, J. W. "The Strauss test for hepatic insufficiency." Bull. Johns Hopkins Hosp., 1912, XXIII, 10.

SACCHAROSE

Saccharose is seldom encountered in the urine. It is dextrorotatory. After inversion (heat 75 c. c. of the urine with 5 c. c. conc. HCl for five minutes at a temperature between 68 and 70° C.), the fluid becomes levorotatory, since the levulose which is formed more than neutralizes the glucose. Reduction tests become positive after inversion of the sugar.

PENTOSE

Pentose is rarely found in the urine. Pentoses are sugars with five carbon atoms. The only one of importance in the urine—r-arabinose—is, unlike other sugars, optically inactive. It reduces copper and bismuth solutions slowly and incompletely; with Nylander's solution a grayish precipitate may be obtained. Pentose does not ferment with yeast. Pentose should be suspected when the reduction tests are atypical, when they persist after attempts at fermentation, when the urine is inactive on polariscopic examination. The following tests may also be employed:

(1) **The Phloroglucin Test.**—To about 5 c. c. of urine in a test tube add an equal volume of concentrated hydrochloric acid and a liberal knife-point (ca. 30 mg.) of phloroglucin. The mixture is heated, preferably on a water bath. A red color appears, and, soon afterward, a dark precipitate forms. The contents of the test tube are cooled, and are then extracted with amyl alcohol. Spectroscopic examination of the amyl alcohol extract reveals a band midway between D and E, a little to the right of the sodium line.

Sources of Error.—Glycuronic acid compounds yield a positive phloroglucin test, including the absorption band, thus lessening greatly the value of the test. Lactose and

galactose give the same color reaction as pentose, but the characteristic absorption spectrum is lacking.

(2) **The Orcin Test.**—Equal parts of the urine and concentrated hydrochloric acid (sp. gr. 1.19) and a small knife-point of orcin are boiled gently. If pentose is present a dark greenish color soon develops, and, finally, a turbidity, due to a dark blue or green precipitate. The contents of the test tube are cooled, until they are *lukewarm,* and are then extracted with amyl alcohol. The latter exhibits a dark, olive-green color, the depth of which depends largely upon the concentration of pentose in the urine. If the fluid is cold instead of lukewarm when extracted, the amyl alcohol is reddish and the absorption bands are not so plainly visible (Salkowski). Spectroscopic examination reveals a band at D, the sodium line.

Sources of Error.—The orcin test is also given by the paired glycuronic acid compounds. However, the latter react with orcin less readily than with phloroglucin, so that of the two the orcin test is to be preferred. It has been shown [1] that filter paper may contain pentose-like substances, which are soluble in hydrochloric acid. The urine should, therefore, not be passed through filter paper. Glass wool or asbestos should be employed in its stead.

(3) **Bial's Modification [2] of the Orcin Test.**

Reagent:

Orcin	1.0	gm.
30 per cent. hydrochloric acid.....	500.0	c. c.
10 per cent. ferric chlorid.........	25	drops

Keep the reagent in a dark bottle.

[1] Umber, F. "Notiz über Pentosenreactionen in filtrirten Flüssigkeiten." *Berlin. klin. Wchnschr.*, 1901, XXXVIII, 87.

[2] Biál, M. "Ueber die Diagnose der Pentosurie mit dem von mir angegebenen Reagens." *Deutsche med. Wchnschr.*, 1903, XXIX, 477.

Method.—Heat about 4 c. c. of the reagent to boiling and then add a few drops of the urine to be tested. With pentose a green color develops immediately or in a few seconds. The quantity of urine employed should not exceed 1 c. c. Performed in this way, the test reacts only with pentose, not with paired glycuronic acid compounds (Bial).

The green fluid is extracted with amyl alcohol and examined spectroscopically, as in the orcin test.

The specificity of the test has been questioned by a number of observers.

GLYCURONIC ACID

Glycuronic acid (glucuronic acid) does not appear as such in the urine, but becomes paired or conjugated in the body with various substances, such as indoxyl, skatoxyl, phenol, in which form it is excreted in the urine. In small quantity it is normally met with. Glycuronic acid also combines with numerous drugs. Urochloralic acid, the chloral hydrate compound, is an example. When present in considerable amount, the glycuronates may lead to difficulty in analysis, since many of their reactions resemble those of glucose and other carbohydrates.

With copper solutions the glycuronic acid compounds may give a slow, atypical reduction, often a greenish-yellow precipitate—a reaction quite like that produced by pentose or by very weak solutions of dextrose. The Nylander test may be positive. The phloroglucin and orcin tests are given by the combined glycuronates. The glycuronates do not give the phenylhydrazin test of Cippolina, though the test may become positive if the urine be boiled previously with 1 per cent. sulphuric acid to liberate glycuronic acid;

the crystals obtained melt at 114° to 115° C. The combined glycuronates do not ferment with yeast. It happens, therefore, that, when the urine contains abnormal quantities of both glucose and glycuronates, fermentation does not cause a complete loss of reducing power. The glycuronic acid compounds are levorotatory in acid urine (inactive if the reaction is alkaline), whereas glycuronic acid itself is dextrorotatory. Therefore, boiling one to five minutes with 1 per cent. sulphuric acid changes a levorotation to dextrorotation, or, if glucose be present, it may increase the dextrorotation, provided some of the sugar is not destroyed. Pentose (r-arabinose) is optically inactive. β-oxybutyric acid is levorotatory. To distinguish between the levorotation produced by this acid and that due to the combined glycuronates, the urine is precipitated with subacetate of lead and filtered. The glycuronates are precipitated, while β-oxybutyric acid appears in the filtrate, where its presence may be indicated by polariscopic examination. Or, the β-oxybutyric acid may be extracted by shaking the urine with ether three or four times, the glycuronates remaining in the urine.

Normal urine may contain enough levorotatory substances to produce 0.1 degree of levorotation; when the glycuronates are increased, the levorotation is 0.2 degree or more.

B. Tollens' Test.[1]—To 5 c. c. of urine in a test tube add a bit of naphthoresorcin about the size of a millet seed and then 5 c. c. of concentrated hydrochloric acid (sp. gr. 1.19). Boil the mixture gently about one minute, and set the tube aside for about four minutes. Now cool the contents of the

[1] Tollens, C. "Ueber den Glykuronsäuren Nachweis durch die B. Tollensche Reaktion mit Naphthoresorcin und Salzsäure." *München. med. Wchnshr.*, 1909, LVI, 652.

tube under running water. Extract with an equal volume of ether. (The separation of the ether may be hastened by the addition of a few drops of alcohol.) If glycuronates are present in the urine in excess, the ether extract is dark blue to violet, while with smaller amounts a faint bluish or reddish violet color is obtained. Examined spectroscopically, the ether extract shows a single dark band near the sodium line. (The examination should be made at once,[1] as the substance giving rise to the band is not stable.) In place of naphthoresorcin in substance, 0.5 c. c. of a 1 per cent. alcoholic solution of naphthoresorcin may be substituted. The test is sufficiently delicate to detect the small quantities of glycuronates present in normal urine.

The dark pigments formed in this reaction by pentoses and other sugars are insoluble in ether.

ALKAPTONURIA

Alkaptonuria is a very rare condition, a disturbance in metabolism. Of the alkapton bodies, two, homogentisinic acid and uroleucinic acid, have been isolated. When present in the urine they may give to it the following characteristics: The fresh urine is markedly acid. It is normal in color when voided, but on standing oxidation quickly changes the color to a reddish-brown and, finally, to a black. The color changes occur more rapidly when the reaction of the urine is alkaline. The urine reduces copper and silver (the latter in the cold) but not bismuth. The urine does not give the phenylhydrazin test, does not rotate the plane of polarized light, and is not fermentable.

[1] Brooks, B. Personal communication.

ACETONE

Acetone, a ketone, occurs in normal urine in amounts as high as 10 mg. in twenty-four hours. It is a colorless, odorless liquid, very volatile, of low specific gravity.

In testing the urine for acetone, it is usually necessary to distill the specimen. Occasionally, when very large quantities of acetone are present, positive reactions for acetone may be obtained by testing the urine directly. But in no case do such tests, when negative, exclude an acetonuria. When tests of the urine are negative, it becomes necessary to distill a portion of it and *to apply the tests for acetone to the distillate.*

Between 200 and 300 c. c. of urine are acidified with 1 to 2 c. c. of concentrated hydrochloric acid[1] and distilled. The greater part of the acetone is contained in the first 20 or 30 c. c. of the distillate, which is used for the tests to be described. If distillation of the urine is impossible, about 50 c. c. of urine are extracted with 20 c. c. of ether in a separating funnel. The urine is then allowed to escape, and to the ether about 10 c. c. of water are added. The fluids are well shaken. A large part of the acetone is in the water, which is then used for the qualitative tests.[2]

Qualitative Tests

(1) **Gunning's Test.**—Five c. c. of the distillate are rendered alkaline with 5 to 10 drops of ammonium hydrate,

[1] Phosphoric acid may be used. The acid is added only to prevent the distillation of ammonia and excessive foaming of the urine.

[2] Bohrisch, P. *Pharm. Zentralhalle,* 1907, XLVIII, 5; 184; 206; 220; 245. Cited by F. N. Schulz in Neubauer-Huppert's *Analyse des Harns,* 11th Ed., p. 252. Wiesbaden, 1910.

and then Lugol's solution (potassium iodid, 6 gm., iodin, 4 gm., distilled water to 100 c. c.) or tincture of iodin is added until the deep black precipitate which forms no longer dissolves at once. This gradually disappears and is replaced by a yellow precipitate of iodoform crystals, recognized by their characteristic odor and morphology. The crystals are often so small that the high power dry objectives of the microscope are required. They are hexagonal plates, often clustered in the form of six-pointed stars. When atypical, the crystals should be recrystallized from alcohol-free ether. They are colored yellow. When the test is applied directly to the urine the phosphates are precipitated by the ammonia, usually in the form of crystals resembling fern leaves. With very small quantities of acetone it may be necessary to wait twenty-four hours for the crystals of iodoform to form.

This test is the best qualitative test, since a positive reaction is obtained only with acetone. It is slightly less sensitive than Lieben's test. According to Bohrisch, the test should be applied only to the distillate, not to the urine directly.

(2) **Lieben's Test.**—A few drops of sodium or potassium hydrate and then a little Lugol's solution are added to about 5 c. c. of the distillate, and the mixture is warmed. With large quantities of acetone, an immediate precipitation of yellow iodoform crystals (hexagonal plates or six-pointed stars) occurs. When the amount of acetone is small (0.01 mg. or less), a few hours may be required for the formation of the crystals, which are detected by microscopic examination of the sediment. Warming the tube intensifies the characteristic iodoform odor. The test is very delicate. Crystals may be demonstrable after twenty-four hours with as little as 0.0001 mg. of acetone. If the crys-

tals are atypical, the precipitate is dissolved in alcohol-free ether and recrystallized.

Sources of Error.—Both alcohol and aldehyde give Lieben's test.

(3) **Legal's Test.**—A few small crystals of sodium nitroprussid are dissolved in about 5 c. c. of the distillate. An excess of sodium or potassium hydrate is now added. If acetone is present, a red color develops, which soon changes to yellow. Glacial acetic acid, added in excess while the color is still red, causes a change to purplish red and finally to violet. The test indicates about 0.1 per cent. of acetone.

Sources of Error.—According to v. Jaksch, paracresol gives a yellowish-red color with sodium nitroprussid and sodium hydrate; on adding an excess of glacial acetic acid the color changes to a rose red, and may be confused with the acetone reaction. Creatinin causes the same preliminary color changes as acetone, but on acidifying with glacial acetic acid the color gradually becomes green and then blue. When testing the distillate this difficulty is removed.

(4) **Lange's Test.**[1]—About 15 c. c. of urine are placed in a test tube and treated with 0.5 to 1 c. c. of glacial acetic acid. After the addition of a few drops of a freshly prepared solution of sodium nitroprussid, ammonium hydrate is carefully layered above the urine. In the presence of acetone an intense violet ring appears at the line of contact. The quantity of nitroprussid used is unimportant, but the amount added should not be enough to color the urine. The test, which is a modification of Legal's, is sensitive to acetone in 1/400 per cent. solution. The reaction is not given by alcohol or aldehyde.

[1] Lange, F. "Eine Ringprobe auf Azeton." *München. med. Wchnschr.*, 1906, LIII, 1764.

DIACETIC ACID

Diacetic acid or acetoacetic acid, the precursor of acetone, is usually found in urines which contain abnormal amounts of acetone. When urine is allowed to stand the diacetic acid soon becomes converted into acetone, which in turn is lost within a few hours through volatilization or decomposition. Diacetic acid may, however, be kept in the urine with little loss for weeks by the addition of toluol to the specimen in a tightly stoppered bottle. Unless toluol be added, the urine should be tested for diacetic acid soon after it is voided.

(1) **Gerhardt's Test.**—To about 20 c. c. of urine in a test tube add an excess of 10 per cent. ferric chlorid solution. If a precipitate forms, it is removed by filtration. To the filtrate more ferric chlorid is added, as long as it produces a perceptible darkening in color. A deep Bordeaux red color is produced by diacetic acid. The contents of the test tube are now halved, one portion being boiled, the other set aside as a control. If the color be due to diacetic acid boiling for several minutes (two or more) lessens its intensity very perceptibly, owing to the breaking up of the diacetic acid.

The test indicates 0.04 to 0.05 per cent. of diacetic acid.

Sources of Error.—After the administration of various drugs, notably salicylic acid, aspirin, diuretin, salol, phenacetin, acetates, formates, etc., a red color, at times indistinguishable from that produced by diacetic acid, may be seen. Except in the case of formates and acetates, the color *does not fade* after boiling a few minutes or standing several hours, as is the case when it is due to diacetic acid. When both diacetic acid and one of these drugs coexist, the urine is distilled, and the distillate tested for acetone.

When the disturbing body is either a formate or an acetate, the urine is acidulated with sulphuric acid, cooled if necessary, and then extracted with an equal volume of ether. With a pipette the ether is transferred to another test tube and a small quantity of very dilute watery ferric chlorid solution added; the tube is then well shaken. Diacetic acid causes a violet color in the watery layer, which changes to a Bordeaux red on the addition of more ferric chlorid. The color fades quickly on boiling the watery layer (remove ether first!). Formates and acetates do not give this reaction.

(2) **Arnold's Test.**[1]

Reagents:

Solution A.—1 gm. of paramidoacetophenon is dissolved in 80 to 100 c. c. of distilled water with the aid of hydrochloric acid added drop by drop during vigorous shaking. Acid is added till the yellow solution becomes water clear. An excess of hydrochloric acid is to be avoided.

Solution B.—Sodium nitrate, 1 per cent. aqueous solution.

The solutions keep well.

Method.—Two parts of solution A are mixed with 1 part of solution B (always prepare freshly at the time of making the test). Add an equal volume or less of the suspected urine and then 2 to 3 drops of strong ammonia, shaking well. All urines give a more or less intense brownish-red color. With excessive quantity of diacetic acid the addition of the ammonia produces an amorphous, brownish-red precipitate, but with smaller amounts no precipitate forms. A portion of the reddish fluid is placed in a wine glass or test tube, and a great excess of concentrated

[1] Arnold, V. "Eine neue Reaktion zum Nachweis der Acetessigsäure im Harn." *Wiener klin. Wchnshr.*, 1899, XII, 541.

hydrochloric acid is added (to 1 c. c. of fluid add about 10 to 12 c. c. HCl). In the presence of diacetic acid the mixture takes on a beautiful purplish-violet color. With large amounts of diacetic acid the violet predominates, while with smaller quantities the red is more evident. With normal urine (free of diacetic acid) only a yellow color is obtained.

With small amounts of diacetic acid the reaction may fail if the urine be highly colored. In such case filter the urine through animal charcoal, and the reaction becomes positive with the water-clear filtrate. In using the filtrate add 2 to 3 parts of filtrate to 1 part of the mixed reagent.

The reaction is specific for diacetic acid and its ethyl ester. It is not given by drugs and is more delicate than Gerhardt's test (Arnold).

β-OXYBUTYRIC ACID

β-oxybutyric acid, the third of the "acetone bodies," is found in the urine only in the presence of acetone or diacetic acid, or both, though the converse of this is not true. It occurs in largest amount in certain cases of diabetes mellitus. Its presence may be suspected when the urine is found to be definitely levorotatory after fermentation of the glucose; such a finding is not, of course, conclusive evidence of the presence of this body.

(1) **Black's Test.**[1]—Five or 10 c. c. of urine are concentrated in an evaporating dish at a gentle heat to one-third or one-fourth of the original volume, which eliminates the acetacetic acid. The residue is then acidified with a few drops of concentrated hydrochloric acid, and made to a thick paste with plaster of Paris and allowed to stand until

[1] Black, O. F. "The detection and quantitative determination of β-oxybutyric acid in the urine." *Jour. Biol. Chem.*, 1908, V, 207.

it begins to set. It is then stirred and broken up in the dish with a blunt stirring rod. The porous meal thus obtained is extracted twice with ether by stirring and decantation. The ether extract, which contains β-oxybutyric acid, is evaporated spontaneously or on the water bath. The residue is finally dissolved in water and neutralized with barium carbonate. The fluid is now poured into a test tube and treated with 2 or 3 drops of commercial hydrogen peroxid, the whole being mixed by shaking. The β-oxybutyric acid is oxidized to diacetic acid. Now add a few drops of 5 per cent. ferric chlorid containing a trace of ferrous chlorid. On standing a few seconds a beautiful rose color develops, which slowly intensifies until it reaches a maximum, and then gradually fades, owing to the further oxidation of the acetacetic acid.

Sources of Error.—Black says that the chief precautions to be observed in carrying out the test are to be sure that the solution is cold and nearly neutral, and to avoid a large excess of hydrogen peroxid and iron. If too much of the oxidizing agents is added, and but little β-oxybutyric acid is present, the color developed is transitory or fails to appear. By starting with a small quantity and then adding more ferric chlorid at intervals of a few minutes, until no further color is produced, one is able to observe the full intensity of color, and thereby get a rough idea as to the amount of β-oxybutyric acid present.

The test is delicate. Black found that a solution containing 0.1 mg. per cubic centimeter, or one part in 10,000, gave an easily recognized color.

(2) **Hart's [1] Test.**—Hart adds to 20 c. c. of the suspected urine 20 c. c. of water and a few drops of acetic acid, and

[1] Hart, T. S. "The detection of β-oxybutyric acid in the urine." *Amer. Jour. Med. Sc.*, 1909, CXXXVII, 869.

boils, until the volume is reduced to about 10 c. c. To this residue add water to the original volume, 20 c. c. Put this into two test tubes (B and C) of equal size, 10 c. c. in each test tube. To one of the test tubes (C) add 1 c. c. of peroxid of hydrogen, warm gently for about one minute (do not boil), and then allow the fluid to cool. Add to each test tube 0.5 c. c. of glacial acetic acid and a few drops of a freshly prepared solution of sodium nitroprussid, and mix. Overlay the solution in each test tube with 2 c. c. of ammonium hydroxid (Lange's test, p. 64). Allow the tubes to stand for four or five hours, and at the end of this time compare them. At the point of contact between the ammonia and the underlying fluid, B will show no ring (or a faint brown ring, if much creatinin is present); test tube C, to which the hydrogen peroxid was added, will show a purplish-red contact ring, if β-oxybutyric acid was originally present; if β-oxybutyric acid was not present the two test tubes will not differ in appearance. If the two tubes are now shaken the difference in color will be seen throughout the fluid; this difference is intensified by allowing the tubes to stand for fifteen or twenty minutes after shaking.

The oxidation of the β-oxybutyric acid to acetone by means of the hydrogen peroxid is said to be gradual, and reaches its maximum in about four or five hours, after which the color slowly fades. When a very large amount of β-oxybutyric acid is present the difference in the two tubes may become evident in a few minutes. The two tubes should always be prepared as above. B will show whether all preformed acetone and diacetic acid have been driven off.

The presence of sugar does not interfere with the reaction. If albumin is present, it should be removed.

The method, though simpler than Black's, does not compare with the latter in delicacy. Hart finds that it will certainly detect β-oxybutyric acid when present to the extent of 0.3 per cent. and probably less.

UROBILINOGEN

Urobilinogen is normally present in the urine in traces. It is converted into urobilin within a few hours after the urine is voided, so that it is necessary to employ fresh specimens in testing for it. In the twenty-four-hour specimen an excess of the chromogen, though originally present, may be missed by the time the examination is made; in this case urobilin may be looked for.

Ehrlich's Aldehyde Test.

Reagent: [1]

Dimethylparamidobenzaldehyde	2.0 gm.
Hydrochloric acid (5 per cent.)[2]....	100.0 c. c.

Dissolve. Keep in dark brown glass bottle.

About 10 c. c. of urine in a test tube are treated with a few drops of the reagent. In the presence of abnormally large amounts of urobilinogen a red color develops in the cold. In normal urine the red color appears only after heating. If the color fails to develop on heating, urobilinogen is absent.

UROBILIN

Urobilin, whose chromogen is urobilinogen, is a constituent of normal urine. Though it be lacking in the freshly voided specimen, traces of it are soon present, due to

[1] As the reagent does not keep well, it should be prepared in small quantity, according to the demand.

[2] About 14 c. c. conc. HCl diluted to 100 c. c. with water.

the action of light on urobilinogen. Large quantities of the pigment may impart a deep yellowish-brown color to the urine, though an excess may be present without noticeable change in the appearance of the specimen.

(1) **Spectroscopic Determination.**—When there is a considerable excess of urobilin it may be detected by direct spectroscopic examination of the urine. A small hand spectroscope is most convenient for the purpose. A few c. c. of clear urine, previously treated with a few drops of Lugol's solution (about 1 drop to 2 c. c.), and a few drops of mineral acid are examined directly with the spectroscope. The characteristic spectrum of acid urobilin, a single band (Fig. 7) between the green and the blue parts of the spectrum (between the lines b and F and extending a little to the right of F in the green), is seen. If the urine is very highly colored it may be necessary to dilute it before examining it with the spectroscope. On the other hand, with small amounts of urobilin the pigment should be extracted from the acidulated urine with amyl alcohol. The extract then presents the band of urobilin in acid solution.

The filtrate in the next two tests may also be examined spectroscopically. It must be remembered that urobilin in alkaline solution shows a band between b and F which is nearer b than that seen in acid solution. In solution with ammonia and zinc, the band is well seen. But if alkali be added to the urine for direct spectroscopic examination, fixed alkali will produce a darker band than ammonia.

(2) **Schlesinger's** [1] **Test.**—This is the best test for routine work. It is delicate, easily performed, and requires no special apparatus.

About 10 c. c. of acid urine are treated with 5 or 6 drops

[1] Schlesinger, W. "Zum klinischen Nachweis des Urobilins." *Deutsche med. Wchnschr.*, 1903, XXIX, 561.

of Lugol's solution to convert any urobilinogen present into urobilin. The urine is now mixed with an equal quantity of a saturated solution of zinc acetate in absolute alcohol and filtered. When held against a dark background and examined with transmitted light, the filtrate shows a beautiful green fluorescence, whose intensity is proportional to the quantity of urobilin present. The fluid may be examined spectroscopically.

The least trace of eosin or other fluorescing compound on the glassware may lead to misinterpretation of the result of the test.

The fluorescence may be made to appear more marked if the light be focused on the tube with a small hand lens.

The alcoholic zinc acetate solution precipitates other pigments, which may interfere with the reaction. However, when bilirubin is very abundant it is precipitated by adding 2 c. c. of 10 per cent. calcium chlorid solution to 8 c. c. of urine. The mixture is filtered and the test applied to the filtrate. The test is sensitive to 0.002 per cent. solutions of urobilin in urine, even in the presence of bile pigments.

(3) **Jaffé's Test.**—The urine is treated with a few drops of Lugol's solution and then with an equal volume of 10 per cent. alcoholic solution of zinc chlorid. The precipitate which forms is removed by filtration. The filtrate is rendered strongly alkaline with ammonia. A green fluorescence denotes the presence of urobilin. On spectroscopic examination the spectrum of urobilin in alkaline solution may also be observed. The single band is a little nearer b than that seen in acid solution (Fig. 7).

The test is not as delicate as Schlesinger's test. Zinc chlorid precipitates the interfering bodies less completely than zinc acetate, but alcoholic zinc chlorid is preferable to

the aqueous solution, which is frequently recommended (Schlesinger).

When it is desired to determine whether urobilin is totally absent from the urine, large quantities of urine treated with Lugol's solution and mineral acid are extracted with a small volume of amyl alcohol, which may then be subjected to spectroscopic examination or to Schlesinger's test. The aldehyde test of Ehrlich is equally decisive in proving the absence of the pigment if a perfectly fresh specimen of urine be employed.

BILE PIGMENTS

Bile pigments, never normally present in the urine, may or may not cause an appreciable alteration in its appearance, the result depending on the concentration of the coloring matter. When much is present the urine has a dark brown, at times a greenish-brown, color. The pigments exist in the urine as such or as soluble combinations with the alkalies or alkaline phosphates. Thus it happens that *bilirubin (hematoidin) in crystalline form* is not usually seen in the urine; but in the urine of infants with small content in phosphates such crystals not infrequently form. They appear as yellowish or brownish-red needles or as rhombic plates or prisms, the latter often with rounded angles. The crystals are insoluble in water. In chloroform, especially if hot, they dissolve readily (1:600), imparting their color to the solution. In dimethylanilin bilirubin crystals are very soluble (1:100). The alkaline compounds of bilirubin found in the urine are insoluble in chloroform. Bilirubin may, therefore, be removed from chloroform solution by alkali. The bilirubin as found in

the urine is precipitated by the addition of hydrochloric acid. From ammoniacal solution bilirubin is precipitated by barium chlorid, lead acetate, and silver nitrate.

Solutions of bilirubin possess no characteristic absorption bands; there is a continuous absorption from the red to the violet end of the spectrum.

Qualitative Tests

(1) **Foam Test.**—The urine is shaken vigorously and, if it contains considerable bile pigment, the foam presents a distinct yellow color. The test is practically specific, but is not very delicate. Similarly, icteric urines show a *yellow staining of the sediment,* and a similar color is left on filter paper, through which such urines have been passed.

(2) **Gmelin's Test.**—The acid urine is superimposed carefully on yellow nitric acid [1] in a test tube; the layering of the two fluids must be sharp. In the presence of bilirubin a play of colors is observed at the line of contact of the two fluids. The colors from above downward are green, blue, violet, red, yellow. A piece of white paper, held behind the test tube, with the light at the examiner's back, aids in the recognition of the colors. The green color is the most important. The reaction is said to be positive in a dilution of 1:80,000.

Albumin and urobilin do not interfere with the reaction. Much indican may lead to confusion at times; other tests should then be resorted to.

(3) **Rosenbach's Modification of Gmelin's Test.**—The urine, slightly acidified with hydrochloric acid, is passed

[1] Yellow nitric acid is quickly obtained by adding a small piece of pine or other soft wood to nitric acid. In a short time, nitrous acid, HNO_2, is evolved. The acid should be light yellow; too much nitrous acid may accelerate the oxidation to such an extent that the colors are missed entirely.

through a small filter paper several times. The paper is then unfolded and blotted lightly with dry paper. The stain on the paper is now touched with a drop of yellow nitric acid, when, in the presence of bile pigment, a play of colors is seen at the edge of the drop. From within outward the colors are yellow, red, violet, blue, and green. The green color, the result of oxidation of bilirubin to biliverdin, is again the most important color; in its absence the test is negative. If the paper be allowed to dry, it should be moistened with a drop or two of water before applying the acid. The test is very delicate and practically specific.

(4) **Huppert's Test.**—In deeply pigmented urines or in those rich in indican or hemoglobin, this test is preferable to Gmelin's. The urine is made alkaline with sodium or ammonium carbonate, and then calcium chlorid solution is added as long as a precipitate forms. The mixture is passed through a small filter, the precipitate washed with water, and the precipitate and filter paper transferred to a test tube or porcelain evaporating dish. Acid alcohol (concentrated hydrochloric acid, 5 c. c., alcohol, 95 c. c.) is now added and carefully heated to boiling. In the presence of bilirubin the color of the alcoholic solution changes to green or blue. The delicacy of the reaction varies between a dilution of bile of 1:500,000 and 1:1,000,000 (Hammarsten).

(5) **Hammarsten's Test.**

Reagent:

Nitric acid (25 per cent.)..........	1 part
Hydrochloric acid (25 per cent.)...	19 parts

The reagent may be kept for about a year. It is not ready for use until its color becomes yellow.

Ordinarily it is sufficient to pour a few drops of urine into 2 or 3 c. c. of the reagent. Almost immediately after shaking, the mixture takes on a green or bluish-green color, which will persist about twenty-four hours. When only traces of bile pigment are present, 10 c. c. of the acid or neutral (not alkaline) urine are treated with a 10 per cent. solution of barium chlorid, which is added as long as a precipitate forms. The mixture is now centrifugalized. The supernatant fluid is poured off and the sediment is shaken with about 1 c. c. of the reagent and again centrifugalized. The supernatant fluid is now a beautiful green, which changes upon further addition of the reagent through blue to violet, red, and finally reddish-yellow. The green color is obtained in the presence of 1 part of bile pigment to 500,000 to 1,000,000 parts of urine. In the presence of considerable amounts of blood-coloring matter or other pigments, a 10 per cent. solution of calcium chlorid should be substituted for the barium chlorid solution.

HEMATOPORPHYRIN

Hematoporphyrin, an iron-free derivative of hemoglobin, is a normal urinary pigment occurring in traces. When urine contains hematoporphyrin in considerable concentration, its color is usually dark red.

Garrod's Test.—One hundred c. c. of urine are treated with 20 c. c. of 10 per cent. sodium hydrate; this precipitates the phosphates, which carry the pigment down with them. The precipitate is collected by filtration or centrifugalization, and is dissolved in acid alcohol (HCl, 5 c. c., alcohol, 95 c. c.). The solution is examined spectroscopically for the bands of hematoporphyrin in acid solution (Fig. 7), one just to the left of D, the other—a broader

band—between D and E. The result may be further controlled by obtaining the absorption bands of hematoporphyrin in alkaline solution. The alcoholic solution is rendered alkaline with ammonia, acetic acid is added till the precipitate of phosphates is dissolved, and the pigment is then extracted with chloroform. The latter is examined spectroscopically for the four bands of hematoporphyrin in alkaline solution (Fig. 7); the first about midway between C and D, the second at D extending to the right of it, the third at the left of E, the fourth—a broad band—beginning at b and extending almost to F.

In place of sodium hydrate, Salkowski recommends that the urine be treated with a solution composed of equal parts of cold saturated barium hydrate and 10 per cent. barium chlorid, while Hammarsten prefers a solution of barium acetate. In either case the precipitate is washed and then dissolved in acid alcohol, as in Garrod's test, and the alcoholic solution is examined for the spectrum of hematoporphyrin in acid solution. By adding an excess of ammonia the bands of alkaline hematoporphyrin are obtained.

In certain instances the urine contains hematoporphyrin in such concentration that direct spectroscopic examination reveals its presence. In such case the alkaline spectrum is the one usually observed, though the spectrum of hematoporphyrin in acid solution may be seen, and is still sharper after acidifying the urine with a mineral acid.

HEMOGLOBIN

Hemoglobin in the urine is always pathological. The hemoglobin is often changed to methemoglobin. If the urine contains hemoglobin in relatively high concentration

it assumes a dark red color, which is imparted to the sediment. The latter, however, is often dark brown. Occasionally crystals of hematoidin are found on examination of the sediment (see bilirubin crystals); when placed in a porcelain dish with a drop of yellow nitric acid, a play of colors, especially a green ring, is seen at the periphery of the drop (Gmelin's reaction). In hemoglobinuria red blood corpuscles are usually absent or present in small number.

(1) **Spectroscopic Determination** (Fig. 7).—The urine, if neutral or alkaline, is rendered slightly acid with dilute acetic acid, and is filtered till perfectly clear. If very highly colored it may be necessary to dilute the specimen before examining it spectroscopically.

(a) *Oxyhemoglobin* is characterized by the appearance of two bands between D and E, the narrower band being near D.

(b) *Reduced hemoglobin* is recognized by a single broad band, extending from D toward E.

(c) *Methemoglobin* in neutral or weakly acid solution produces a dark band in the spectrum between C and D, near C. The two additional bands seen between D and E are usually attributed to the coexistence of oxyhemoglobin in the solution. In alkaline solution methemoglobin presents two absorption bands between D and E, resembling those of oxyhemoglobin, except for the fact that the narrower band in this case is situated at the right.

(d) *Hematin* is found in the urine very infrequently. In acid solution its spectrum resembles closely that of methemoglobin in neutral or acid solution, consisting of a single dark band in the red, extending to the right of C. Hematin is readily differentiated from methemoglobin by the fact that the addition of ammonia and a reducing sub-

stance, such as ammonium sulphid, to the acid solution converts the spectrum into that of hemochromogen.

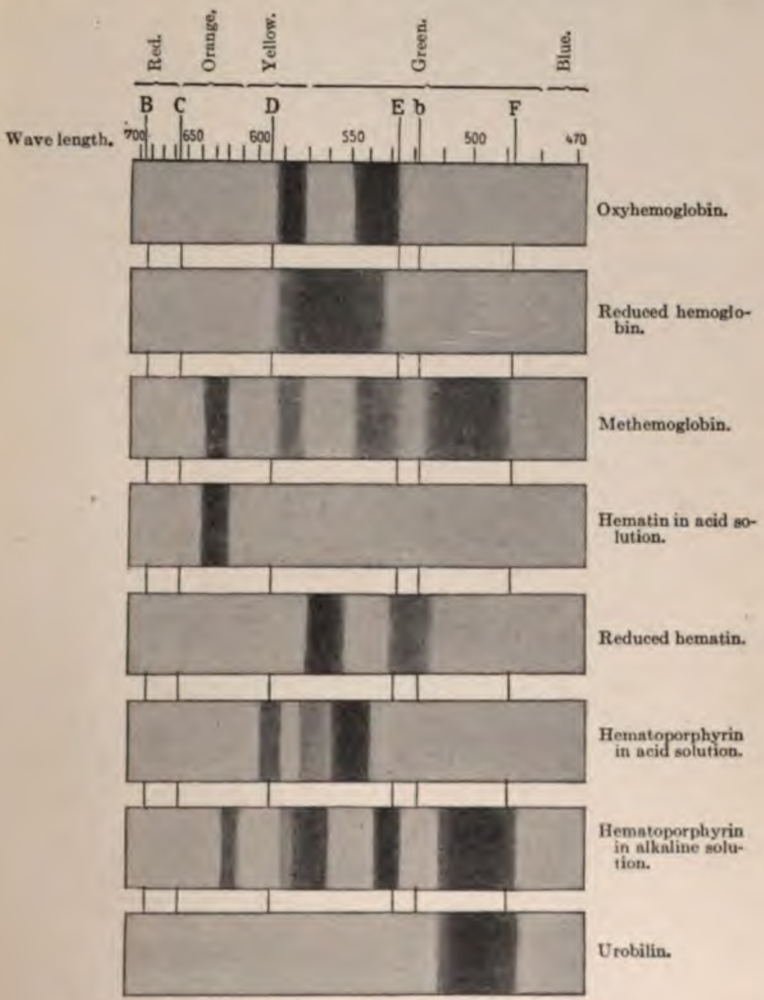


FIG. 7.—ABSORPTION SPECTRA. (After Seifert and Müller.)

(e) *Hemochromogen* [reduced hematin (d)] presents two dark bands, one about midway between D and E,

the other to the right of E. Both bands are nearer the green end of the spectrum than those of oxyhemoglobin.

In case the urine is very deeply pigmented, the *spectroscopic examination* is facilitated by dilution with water, since concentrated solutions absorb the spectrum diffusely. On the other hand, with small amounts of hemoglobin, the delicacy of the spectroscopic test depends very largely upon the thickness of the layer of urine, through which the light passes to the spectroscope. Schumm [1] finds that with the usual test tube hemoglobin may be detected spectroscopically in a dilution of 1:2,000, whereas, if the urine be placed in the polariscope tube 10 or 20 cm. long, it is possible to recognize one part of hemoglobin in about 25,000 parts of urine. Roughly, this is equivalent to one drop of blood in the twenty-four-hour specimen. If the oxyhemoglobin has been changed to methemoglobin, Schumm recommends the following procedure: 50 c. c. of urine, 5 c. c. of glacial acetic acid, and 40 to 50 c. c. of ether are shaken together. The ether, after it has separated, is drawn off and shaken with 5 c. c. of water, which is then removed. The guiac test (p. 81) is then applied to a part of the ether extract. To the remainder add ammonia in excess (keep the mixture cool) and shake well. The ammonia layer and a part of the ether are allowed to run into a glass, ammonium sulphid is added, and the bands of alkaline hemochromogen are looked for.

The following tests are applicable alike to the detection of *hemoglobinuria* and *hematuria:*

[1] Schumm, O. "Untersuchungen über den Nachweis von Blut im Harn mit Hilfe des spektroskopischen und einiger spektroskopisch-chemischer Verfahren." *München. med. Wchnschr.*, 1908, LV, 1488.

(2) **The Guiac Test.**[1]

Reagents:

Guiac resin, powdered.
Alcohol.
Hydrogen peroxid *or* ozonized oil of turpentine.

Tincture of guiac is freshly prepared at the time of making the test by adding a knife-point of powdered guiac to about 5 c. c. of alcohol, shaking till solution occurs.

Equal parts of tincture of guiac and hydrogen peroxid are mixed and are then layered above the urine by inclining the test tube and pouring the mixture in very slowly. The urine, if neutral or alkaline, is acidified with acetic acid before testing. An opaque ring forms at the line of contact between the fluids; gradually a distinct blue color develops.

The test is very delicate, but it is not specific for blood. Disturbing substances are much less apt to be encountered in the urine than in the stools or gastric contents. Pus, when present, gives the blue color, but the reaction occurs without the addition of the peroxid. The urine may be treated with glacial acetic acid and extracted with ether (for details consult p. 159). For a complete list of the disturbing substances the reader is referred to the monograph of Kastle.

This test is very delicate; it may be positive with 1 part of blood in 20,000 to 40,000 parts of urine. It is chiefly of value when negative. A positive test does not mean the presence of blood—it must be confirmed by other tests; but a negative reaction is conclusive evidence of the absence

[1] For a full discussion of this and allied tests, see Kastle, J. H., "Chemical tests for blood." *Bull. No. 51*, U. S. Pub. Health & Mar. Hosp. Serv., Wash., pp. 1-62, 1909.

of blood. The activity of the guiac should be proved occasionally.

Several other chromogenic substances have been used successfully in place of guiac, but they are all open to the same objection, i. e., lack of specificity. *Benzidin, aloin,* and *phenolphthalin* are the bodies most frequently substituted for guiac.

(3) **Heller's Test.**—The urine, if alkaline, is rendered neutral or slightly acid with acetic acid, and is then boiled. If much blood is present, a precipitate of albumin and hematin forms. The hot urine is now treated with sodium or potassium hydrate. The phosphates are precipitated and carry down with them any hematin present. The latter colors the precipitate red, which constitutes a positive reaction. In case the phosphates have already been precipitated from the urine, normal urine may be added to supply the salts in solution; or a little calcium chlorid solution is added to the urine, which is then boiled, and sodium phosphate is poured into it with the sodium hydrate (Hammarsten).

To prove beyond question that the red precipitate is caused by blood pigment, the precipitate is subjected to Teichmann's hemin crystal test.

Heller's test is not very delicate, and is, therefore, less used now than formerly.

(4) **Teichmann's Hemin Crystal Test.**—The precipitate obtained in Heller's test or from treating the urine with tannic acid is used for this test. The excess of phosphates may be removed by washing the precipitate with very dilute acetic acid. The precipitate is then dried on the filter paper, and a small amount of it transferred to a clean glass slide. To it add a few *small* crystals of sodium chlorid. Crush the crystals and mix the powder with the pre-

cipitate. A cover glass is placed on the material, and glacial acetic acid is run under it. Heat the preparation just short of boiling ¾ to 1 minute, replenishing the acid as necessary. The fluid turns brown. The specimen is allowed to cool a few minutes, and is then examined microscopically for the brown rhombic plates of hemin. It is often necessary to reheat the specimen several times before the crystals are obtained. Instead of heating the specimen, it may be set aside for twenty-four hours before examining it; in this case the crystals are usually somewhat larger. With small crystals, high magnification may be required for their recognition.

The test is specific for hemoglobin. It often fails if too much sodium chlorid is added, or if the specimen is overheated.

THE DIAZO REACTION

Ehrlich's diazo reaction is never given by normal urine, but is of frequent occurrence in febrile diseases, comparatively rare in afebrile conditions.

Reagents:

Solution 1:

Sodium nitrite	0.5 gm.
Distilled water	100.0 c. c.

Dissolve. The solution does not keep well.

Solution 2:

Sulphanilic acid	5.0 gm.
Hydrochloric acid, conc	50.0 c. c.
Distilled water to	1,000.0 c. c.

Dissolve.

of solution 1 is mixed with 50 parts of solution ...ture should be prepared freshly each day, as it is not permanent longer than twenty-four hours.

Equal quantities of the mixed solutions and the urine are mixed in a test tube. Ammonium hydrate is then run down the side of the tube, which is inclined, so that it forms a layer at the top. A red ring is formed at the line of contact of the fluids. The test tube is sealed and shaken vigorously. If the foam is colored pink, the reaction is positive. The color fades rather rapidly.

It is quite possible to misinterpret the result of the test, since a salmon or yellowish-red or brownish-red foam is not infrequently observed. It is essential to discard all results as negative unless the foam is unquestionably pink.

At times the bodies causing the diazo reaction exist in the urine in such dilution that a positive reaction is not obtained. In such cases the test again becomes positive, if the urine is concentrated to a small volume on a water bath.

If the sodium nitrite solution is used in greater strength than 1:50, normal urine may give the color reaction. After the administration of atophan (phenyl-quinolin-carboxylic acid), 3 gm. daily, a positive diazo test may be given by normal urine.[1]

CHYLURIA

The admixture of chyle causes the urine to appear more or less milky, depending partly on the proportion of chyle, but still more on its fat content. Whether parasitic (filaria) or non-parasitic in origin, chyluria is probably always

[1] Skorczewski, W., and Sohn, I. "Ueber einige im Atophanharne auftretende charakteristische Reaktionen." *Wiener klin. Wchnschr.*, 1911, XXIV, 1700.

due to a direct anatomical communication between the lymph channels and the genitourinary tract, as Magnus-Levy [1] and others have pointed out. Ureteral catheterization often reveals the fact that the chyluria is unilateral. The symptom is intermittent, as a rule, depending on the posture of the patient; in some cases it appears during the day, in others only at night. All of the normal ingredients of chyle may be found in the urine. They are:

(1) *Neutral Fat.*—Droplets of neutral fat are always present; they are derived from the chyle, not from the blood, and the quantity found varies directly with that ingested in the food. The droplets vary considerably in size; some are so small that they are only seen distinctly with the oil immersion. They possess a sharp contour and are highly refractive. The addition of a drop of Sudan III or of Scharlach R (saturated solution in 70 per cent. alcohol) stains the fat droplets an orange or reddish-yellow color. From the alkaline urine the fat may be extracted by means of the usual fat solvents, such as ether.

(2) *Cholesterin* and *lecithin* are found if large quantities of urine are extracted with ether. Their quantity is dependent largely on the food.

(3) *Sugar* may or may not be discovered in the urine. It has been shown that chyle contains about 0.1 per cent. sugar in hunger or on a fat or protein diet. One part of chyle in two parts of urine under these circumstances would give about 0.03 per cent. glucose—too little to detect with the usual clinical tests. On the other hand, after a large carbohydrate meal, especially a meal containing an excess of sugar, the urine may contain 0.3 to 0.4 per cent.

[1] Magnus-Levy, A. "Ueber europäische Chylurie." *Ztschr. f. klin. Med.*, 1908, LXVI, 482.

of glucose. With this amount glycosuria is easily recognized.

(4) *Lymphocytes* are always to be seen, either in the sediment of the centrifugalized specimen or caught in the meshes of the clots, which occasionally form in the urine.

(5) *Albumin* is generally found in the urine, unless the proportion of chyle be very small. With appropriate tests, such as fractional precipitation of the urine with ammonium sulphate, globulin and fibrinogen may usually be demonstrated.

(6) *Filaria Bancrofti.*—In parasitic chyluria the embryos of Filaria bancrofti are present in the sediment or in the clot (see p. 117).

LIPURIA

Small quantities of fat are not unusual in the urine. When epithelial or pus cells are present it is common to find fat droplets in them, and a few droplets are found free in the urine. The term lipuria is reserved for those conditions in which the fat is so abundant that it is recognized macroscopically. It is important to exclude fat from external sources, such as dirty containers for the urine, the lubricant on catheters, the willful admixture of fat or milk for purposes of deception, etc. Fat is recognized by its appearance and microchemical reactions. Unless it is present in the form of an emulsion, it is seen on the surface of the fluid.

FERMENTS IN THE URINE

A number of enzymes have been discovered in the urine —pepsin, trypsin, lipase, diastase, etc. From a diagnostic standpoint diastase appears to be the most important,

though the determination of lipase has also been of some value in pancreatic disease.

Wohlgemuth's Method for the Determination of Diastase.[1]—A 1 per cent. starch solution is prepared. Merck's or Kahlbaum's soluble starch is employed. The starch powder is stirred in cold distilled water, which is then heated about 10 minutes with constant stirring, until the solution is clear. It is cooled and is then ready for use. Into each of several test tubes 5 c. c. of the starch solution are placed with a pipette. Next add varying fractions of 1 c. c. of urine to the tubes, which have been numbered, beginning with 0.2 c. c. and decreasing gradually. Add a small quantity of toluol to each tube to prevent bacterial growth, and place the tubes in the incubator at 37° C. for twenty-four hours. The tubes are then removed from the incubator and filled almost completely with ice water. To each tube add 1 drop of $\frac{N}{10}$ iodin solution, mix well, and observe for the blue color of the starch-iodin reaction. The first tube which shows no blue is selected. From the known proportions of urine and starch solution in this tube, calculate the number of cubic centimeters of 1 per cent. starch solution which 1 c. c. of urine would convert to dextrin and sugar. Assuming the result to be 150, it is expressed as follows: D $\frac{37^\circ}{24\text{h}}$=150. This means that the urine examined contained sufficient diastase (D) to convert 150 c. c. of 1 per cent. starch solution to dextrin and sugar, acting at 37° C. for 24 hours.

Wohlgemuth employs the urine obtained at the second voiding in the morning for examination. The diastase is

[1] Wohlgemuth, J. (a) "Ueber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments." *Biochem. Ztschr.*, 1908, IX, 1. (b) "Untersuchungen ueber die Diastasen. Beitrag zum Verhalten der Diastase im Urin." *Ibid.*, 1909, XXI, 432. (c) "Beitrag zur funktionellen Diagnostik des Pankreas." *Berlin. klin. Wchnschr.*, 1910, XLVII, 92.

greatest in the urine during fasting, and decreases three to four hours after meals. The highest normal value which he has obtained for the urine is 156.

(2) **Determination of Lipase According to Hewlett.**[1]—Hewlett has adapted the ethyl butyrate method of Kastle and Loevenhart to the determination of lipase in the urine. The procedure follows, in the author's words: Five c. c. of urine are placed in each of three flasks. The urine in the second flask is then boiled. To the urine in the third flask are then added three drops of a 1 per cent. solution of phenolphthalein and tenth normal sodium hydrate is allowed to run in from a burette, until a faint pink color appears throughout the fluid. The amount of sodium hydrate used is read off, and a like amount is added to the first and second flasks. To each of these two flasks, the first of unboiled urine, the second of boiled urine, are then added 0.25 c. c. of ethyl butyrate and 0.1 c. c. of toluene, and they are placed in a thermostat at 38° C. for about 20 hours. The toluene is added to prevent the growth of bacteria. At the end of this time each flask is taken out, and sufficient tenth normal hydrochloric acid is added to more than neutralize the alkali previously added by 0.5 c. c. Each specimen is then shaken in a separating funnel with 50 c. c. of redistilled ether, and the ether is separated. After adding three drops of a 1 per cent. solution of phenolphthalein to 25 c. c. of pure alcohol, the latter is brought to the neutral point. The ether extract from the separating funnel is now added to the neutralized alcohol, and its acidity is determined by titrating with N-20 potassium hydrate solution (alcoholic). Any decided difference between the acidity of the ethereal extracts of the boiled and of the unboiled

[1] Hewlett, A. W. "On the occurrence of lipase in the urine as a result of experimental pancreatic disease." *Jour. Med. Research*, 1904, XI, 377.

urine is due to the butyric acid formed by the cleavage of the ethyl butyrate; and, where the difference in acidity is at all great, the odor of butyric acid can be recognized.

In normal healthy men (and dogs) the urine contains merely traces of lipase. The greatest difference found by Hewlett was 0.35 c. c. of twentieth normal potassium hydrate—usually 0.1 or 0.2 c. c.

THE URINARY SEDIMENTS

The sediment may be obtained for microscopic examination by allowing it to settle in a conical specimen glass [1] or, preferably, by centrifugalizing the urine. The objection to sedimentation is that certain of the formed elements, especially casts, may be more or less completely digested, if the specimen be allowed to stand too long, whereas with a centrifuge the examination may be made almost as soon as the urine is passed. It is particularly in alkaline urines that casts rapidly disappear. It is very important that a perfectly *fresh specimen* of urine be employed for microscopic examination. With urine which has stood for twenty-four hours it is often impossible to gain a correct impression of the sediment. Crystals, which were not present when the urine was voided, may have formed, while the more im-

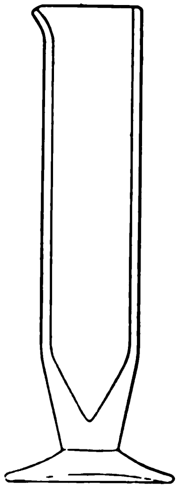


FIG. 8.—THE SYDENHAM SEDIMENTING GLASS.

[1] The most satisfactory sedimenting glass with which the writer is familiar is one designed by Dr. J. S. Brotherhood (see Fig. 8). It permits one to take the specific gravity without transferring the urine to another receptacle, and its shape insures concentration of the sediment at the bottom. The weight of the base is an advantage, as the glass is not easily upset. It may be obtained from the Arthur H. Thomas Co., Philadelphia.

portant organized material may have become so altered that it is no longer recognizable.

The sediment is removed with a pipette and several drops of it are transferred to a glass slide. A cover glass should *not* be placed on the specimen for the preliminary examination, though it may be required later. When the sediment is scanty the few drops of urine adhering to the outer surface of the pipette should be wiped off to prevent dilution of the specimen. On the other hand, with abundant sediment it is often advantageous to thin it somewhat so that the various elements are separated and their recognition made less difficult.

The preliminary examination of a urinary sediment[1] should always be made *with low magnification and with the light cut off as much as possible.* Usually this examination is sufficient. But, if all the elements in the preparation cannot be recognized in this way, a cover glass is placed on the drop of sediment, which is now examined under higher magnification. The oil immersion lens is not employed with a wet specimen, nor is it necessary.

For microchemical tests a cover glass is placed on a drop of the sediment, and any excess of moisture removed with a blotter. With a pipette a drop of the reagent is placed on the slide at one side of the cover glass, while a piece of blotting paper is touched to the opposite side. The absorption of fluid by the paper creates a current, which draws the reagent under the cover glass. The effect upon the sediment is observed with the low power objective. In case it is necessary to use the higher power, great care should be exercised that the lens escapes the reagent.

[1] H. Rieder's "Atlas der klinischen Mikroscopie des Harnes" (Leipzig, 1898) is an extremely valuable and useful reference work on urinary sediments.

The Unorganized Sediments

For convenience the unorganized sediments are divided into those which occur chiefly in acid urine, and those which are encountered mainly when the reaction is alkaline. It must be remembered that the classification is by no means absolute; it frequently happens that a deposit, usually found in an acid urine, persists after the reaction has become alkaline, and, again, that a sediment which is generally met with in alkaline urine may make its appearance while the reaction is still acid.

Sediments in Acid Urine

(1) **The Quadriurates of Sodium and Potassium.**—The quadriurates of sodium and potassium, the "*amorphous urates,*" are chiefly responsible for the pink, salmon-colored, yellow, or reddish deposits which may be seen in an acid urine. The salts are especially apt to be precipitated from concentrated specimens as they become cool. The precipitate absorbs the urinary pigments, urochrome (yellow), and uroerythrin (red). Microscopically, the sediment is finely granular, the granules tending to collect in masses. On heating the specimen over the flame the urates go into solution, but are again precipitated, as the preparation cools. The addition of hydrochloric acid dissolves the deposit; subsequently crystals of uric acid form. The latter are usually colorless. The rapidity with which the uric acid crystals appear varies greatly; often within ten or fifteen minutes they are numerous. Acetic acid also brings the urates into solution, but the formation of uric acid crystals may be somewhat delayed. The quadriurates give a positive murexid test (see p. 9).

(2) **Uric Acid.**—Uric acid may separate from an acid urine from the breaking up of the quadriurates into uric acid and biurates. Crystals of uric acid, usually colored reddish or yellowish-brown, are then deposited, giving rise to the so-called "brick dust" sediment. They may assume a great variety of form when viewed under the microscope. That most frequently encountered is the "whetstone" crystal. It is seen singly or in clusters, often arranged as a rosette. The "church-window" shape is not uncommon. Rhombic plates and six-sided prisms are also characteristic. Hexagonal plates are of less frequent occurrence, and may be colorless; morphologically they are indistinguishable from crystals of cystin. The latter, however, do not give the murexid test. Needles of uric acid arranged in sheaves are rare as a spontaneous sediment, though not infrequently seen after the addition of hydrochloric acid to the quadriurates.

Uric acid crystals are insoluble in acetic and hydrochloric acids. They are unaffected by heating the specimen. The crystals are soluble in sodium or potassium hydrate, and may be reprecipitated by the addition of an excess of hydrochloric acid. They give the murexid test (see p. 9).

(3) **Calcium Oxalate.**—Calcium oxalate crystallizes most frequently in acid urine, but the crystals remain after the reaction has become alkaline. The crystals vary considerably in size; it is often necessary to employ high magnification to recognize them. Most often they occur as small, highly refractive octahedra. Depending upon the position of the octahedron, its form resembles a square envelope or a lozenge, with lines connecting the opposite angles. Dumbbell or hour-glass forms, at times with radial striations, and spheroidal masses constitute rarer

shapes of calcium oxalate. The crystals are usually colorless, but in icteric urine they may be stained yellow. Calcium oxalate crystals dissolve in hydrochloric or other mineral acid, but are insoluble in acetic acid. The envelope forms may be mistaken for triple phosphate; the latter, however, are readily soluble in acetic acid. Calcium sulphate, calcium carbonate, and uric acid may assume the hour-glass form. Microchemical tests serve to differentiate them from calcium oxalate. (1) Calcium sulphate is insoluble in hydrochloric acid. (2) Calcium carbonate dissolves in acetic acid with the evolution of bubbles of carbon dioxid, which may be seen under the cover glass. (3) Uric acid is insoluble in hydrochloric acid and gives the murexid test.

(4) **Calcium Sulphate.**—Calcium sulphate (gypsum) is a rare deposit in very acid urine. It occurs in the form of long, thin, colorless needles, as long, colorless prisms, often arranged in clusters, or as dumbbells or hour-glass crystals. Calcium sulphate is insoluble in mineral acids and in ammonia.

(5) **Monocalcium Phosphate.**—Monocalcium phosphate, acid calcium phosphate, slender, colorless, rhombic tablets, usually in clusters, resembling somewhat calcium sulphate, is, like the latter, of rare occurrence, and is found in very acid urine. The two are easily distinguished by the solubility of the phosphate in acetic acid and in mineral acids.

(6) **Hippuric Acid.**—Hippuric acid crystals are also very rare in the urinary sediment. They are seen as colorless, transparent, four-sided prisms, or as needles. They are insoluble in hydrochloric acid, which distinguishes them from triple phosphate. From uric acid, which they may resemble somewhat, the crystals are differentiated by the

8

fact that they do not give the murexid test, and that they are soluble in alcohol and ether.

(7) **Cholesterin.**—Cholesterin crystals may be found in the urine occasionally. They present a characteristic shape, being rhombic plates, often superimposed, with the acute angle notched, as a rule. On the addition of strong sulphuric acid (concentrated sulphuric acid, 5 parts, water, 1 part), the crystals are stained carmine, which later changes to violet. On adding the sulphuric acid with a little Lugol's solution the play of colors is violet, blue, green, and red. The crystals are soluble in ether.

(8) **Xanthin.**—Xanthin crystals have been observed in human urine in only a few cases. They are colorless and, from their shape, may be mistaken for uric acid. They differ from the latter in that they are soluble on heating. They also dissolve in ammonia and give the xanthin test.

Weidel's Test.—On the water bath the crystals are evaporated to dryness in a porcelain dish, to which chlorin water and a trace of nitric acid have been added. The residue, when exposed to ammonia fumes, is stained reddish or purplish-violet.

(9) **Hematoidin.**—Hematoidin (*bilirubin*) crystals may be found in icteric urine, particularly when it is very acid. They are differentiated from uric acid by the reactions given on page 73. With the murexid test they give a negative reaction. Hematoidin occurs in amorphous masses or as needles, often gathered together to form sheaves, or as rhombs, colored yellow or yellowish-brown.

(10) **Tyrosin.**—Tyrosin has been found in very few instances as a spontaneous urinary sediment. It is found precipitated in the form of needles gathered together in bundles like sheaves of wheat. In impure state tyrosin may

resemble somewhat spherules of leucin. The crystals are soluble in alkali, in ammonia, and in mineral acids, very slightly soluble in acetic acid. From ammoniacal solution tyrosin crystallizes spontaneously on evaporation.

To obtain tyrosin from the urine [1] the twenty-four-hour specimen is treated with neutral, then with basic, lead acetate, as long as a precipitate forms. The excess of lead in the filtrate is removed by precipitation with hydrogen sulphid. The filtrate is now concentrated to small volume on a water bath. By fractional crystallization tyrosin and leucin (which usually coexist) are separated, since it is chiefly the tyrosin which forms the crystalline deposit. The crystals are then subjected to the tests for tyrosin given below. The leucin which is in the filtrate is converted into its copper salt by boiling with freshly precipitated copper hydroxid. From the hot solution it crystallizes in the form of rhombic plates. The crystals are slightly soluble in water, insoluble in methyl alcohol.

Piria's Test.—Dissolve the crystals of tyrosin in warm, concentrated sulphuric acid, permit the solution to cool, then dilute with water, and, finally, neutralize with barium carbonate. The mixture is then filtered, and to the filtrate ferric chlorid solution is added. A violet color appears. The test may fail if free mineral acid remains or if an excess of ferric chlorid be added.

Mörner's Modification of Denigès' Test.—To a few c. c. of a reagent (consisting of 1 volume of formalin, 45 volumes of water, and 55 volumes of concentrated sulphuric acid) add the tyrosin crystals or solution and boil. A beautiful green color develops.

[1] This and the following tests for tyrosin are taken from Hoppe-Seyler's "Handbuch der chemischen Analyse" (H. Thierfelder), Berlin, 1909, pp. 625 *et seq.*, 8th edition.

fmann's Test.—A few crystals of tyrosin are placed in a test tube partly filled with water, to which a few drops of Millon's reagent (one part of mercury dissolved in two parts by weight of nitric acid, sp. gr. 1.42, then warm gently, add two volumes of water, and, after standing several hours, obtain the clear supernatant fluid) have been added. On boiling the fluid is stained a beautiful red, and a red precipitate forms.

(11) **Leucin.**—Leucin is not seen as a spontaneous sediment in the urine. It is usually present with tyrosin, and may separate as globules resembling fat if the urine be concentrated on a water bath. The globules, unlike those of neutral fat, are insoluble in ether. They are usually stained brown and present radial striations or concentric rings, or are hyalin.

To obtain leucin from solution in the urine, see page 95. For its recognition the reader is referred to works on biological chemistry.

(12) **Cystin.**—Cystin, a sulphur-containing amino-acid, occurs in the urine in the form of colorless, hexagonal plates. The presence of the crystals constitutes cystinuria, the manifestation of a rare disturbance of intermediary protein metabolism. (The crystals are usually found in neutral or alkaline urine, but may be considered here for the sake of convenience.) Oftentimes the crystals are superimposed or overlap one another. Uric acid at times assumes the identical crystalline form and may be colorless. The two may be distinguished by the fact that cystin is readily soluble in hydrochloric acid and ammonia; further, by the fact that the murexid test is not given by cystin. Cystin crystals are insoluble in acetic acid, alcohol, and ether. When the crystals are atypical they may be reprecipitated from ammoniacal solution by the addition of

acetic acid. Microscopic examination should then reveal characteristic crystals.

To isolate cystin in solution the urine is treated with neutral, then with basic, lead acetate (see under tyrosin, p. 95). The filtrate is concentrated on a warm bath. Cystin separates on prolonged standing or after the addition of an excess of acetic acid.

Qualitative Test.—Boil a portion of the urine with sodium or potassium hydrate and lead acetate. A black color arises from the sulphid of lead which is formed. Albumin or other proteins, if present, must first be removed.

Sediments in Neutral or Alkaline Urine

In addition to the crystals described in acid urine, which frequently persist after the reaction has become alkaline, there are a number which are commonly found in alkaline urine.

(1) **Tricalcium and Trimagnesium Phosphates.**—Tricalcium and trimagnesium phosphate, the *amorphous* phosphates, are recognized as white or grayish-white deposits, often very abundant, which are easily soluble in hydrochloric and acetic acids. The lack of coloration and the fact that they do not dissolve on heating the preparation differentiate them from the quadriurates, which they resemble somewhat microscopically. The murexid test is negative, a further differential point.

(2) **Calcium Carbonate.**—Calcium carbonate is also usually amorphous, and is generally found mixed with the amorphous phosphates. It differs from the phosphates in the fact that the addition of acid causes solution with the evolution of carbon dioxid. The salt may also appear as

dumbbells or spheres with radiating lines, resembling similar forms of calcium oxalate, calcium sulphate, and uric acid. Its solubility in acids with gas formation identifies it as calcium carbonate.

(3) **Ammonio-magnesium Phosphate.**—Ammonio-magnesium phosphate, "triple" phosphate, is the crystal most commonly observed in alkaline urine. For its formation it is necessary that ammonia be produced. It therefore happens that the crystals are occasionally encountered while the reaction of the urine is still acid, though their number rapidly increases with the progress of ammoniacal fermentation. The crystals belong to the rhombic system. The "coffin-lid" is the commonest form. Erosion of these produces the irregular X-shaped crystals. With good illumination triple phosphate crystals have a greenish tint. They vary greatly in size; at times they are so large that they are visible with the unaided eye. Some of the smallest crystals, when perfect, resemble somewhat the envelope forms of calcium oxalate. Their solubility in acetic acid is a differential point. When the phosphates are precipitated artificially with ammonia, fern-like crystals are usually found. In a native sediment, particularly when it has stood for some time, it is customary to find the majority of the crystals imperfect.

(4) **Ammonium Biurate.**—Ammonium biurate, like triple phosphate, is deposited only as a result of the liberation of ammonia in the urine. It forms balls or spheres of yellow or light brownish color, often with striations, oftener with horny projections or processes, producing the so-called "thorn-apple" or "morning star" crystals. Their shape may be anything, depending on the number, position, and length of the projections. The crystals are soluble in acetic and hydrochloric acids, with the subsequent

formation of uric acid crystals. They give the murexid test.

It is not uncommon to find amorphous phosphates and carbonates, triple phosphate, and ammonium biurate combined in the sediment of an ammoniacal urine.

(5) **Neutral Magnesium Phosphate.**—Neutral magnesium phosphate, dimagnesium phosphate, is a very rare sediment, which is met with in weakly alkaline urine. It forms long, refractive, rhombic plates. On treating it with 20 per cent. ammonium carbonate solution the crystals become opaque and the edges eroded. They are easily dissolved in acetic acid, and may be reprecipitated by the addition of sodium carbonate.

(6) **Neutral Calcium Phosphate.**—Neutral calcium phosphate, dicalcium phosphate, is very infrequently met with in weakly acid, neutral, or weakly alkaline urine. It gives rise to colorless wedges or prisms, usually clumped together. The crystals are soluble in acetic acid. On treating them with 20 per cent. ammonium carbonate, balls of calcium carbonate are produced.

The Microchemical Reactions of Sediments to Reagents

The reactions described above may be summarized as follows:

(1) *Strong acetic acid* dissolves calcium and magnesium phosphates, ammoniomagnesium phosphate, and calcium carbonate, the last with the evolution of gas. It does not dissolve calcium sulphate, calcium oxalate, uric acid, cystin, tyrosin (very slightly soluble), and xanthin. Salts of uric acid are slowly eroded, and after several hours crystals of uric acid are deposited.

(2) *Hydrochloric acid* dissolves all crystals excepting uric acid, hippuric acid, and calcium sulphate.

(3) *Ammonium hydrate* dissolves cystin, tyrosin, and xanthin. Uric acid crystals are partially eroded with the formation of ammonium biurate. Calcium phosphate, calcium sulphate, and calcium oxalate, and the salts of uric acid are unaffected by ammonia.

(4) *Water* in large amount dissolves calcium sulphate; but many other crystals are not wholly insoluble in water—uric acid and its salts, triple phosphate, tyrosin, and xanthin.

(5) *Alcohol* dissolves tyrosin, leucin, cystin, and hippuric acid.

(6) *Chloroform* dissolves bilirubin (hematoidin) and fat.

The Organized Sediments

(1) **Epithelial Cells.**—Epithelial cells are normally found in the urine, due to the fact that the cells of the genito-urinary mucosæ are constantly desquamating. As a rule, the cells are few in number and the majority of them may be caught in the mucous threads of the nubecula, if the specimen be allowed to stand a short time. In the case of women, however, the urine frequently contains a macroscopic sediment composed largely of enormous numbers of epithelial cells, derived chiefly from the vagina.

A variety of form may be noted in the epithelial cells of the urine. The vaginal cells are rather large, squamous cells with relatively small, round or oval nuclei. Sheets of these cells are often shed *en masse.* Cells derived from the kidney are usually round or cuboidal, with large, vesicular nucleus.

The protoplasm of the epithelial cells is prone to under-

go fatty degeneration. The microscopic appearance is fairly characteristic. The droplets differ in size and may be few or numerous; at times the cell is completely filled, and it may be impossible to demonstrate the nucleus. The fat droplets are stained a deep orange with Sudan III or Scharlach R.

Occasionally myelin or albuminous granules are present in the protoplasm.

Since it is quite generally agreed that it is impossible, from their morphology, to determine the origin of epithelial cells seen in the urine, detailed description of them is superfluous.

Epithelial cells are distinguished from pus cells by the shape of the nucleus. The epithelial cell possesses a single round or oval nucleus; rarely, in disease, isolated multinucleated cells are observed. The pus cell, on the other hand, has a polymorphous nucleus. In the fresh sediment the nuclei are not easily seen; they stand out sharply after the addition of dilute (3 per cent.) acetic acid. Staining is somewhat less satisfactory.

"Heart-failure" cells have recently been described in the urine.[1] Like those seen in the sputum, they are epithelial cells, which are laden with altered blood pigment. The pigment granules are light golden yellow in color. At times there is a diffuse yellowish staining of the cells in the absence of icterus. The cells are not uncommon with chronic passive congestion of the kidneys, but they are not diagnostic of the condition; they *may* be found in hematurias unassociated with passive congestion.[2] The cells are usually more or less swollen, of varying size, often very

[1] Bittorf, A. "Ueber Herzfehlerzellen im Harne." *München. med. Wchnschr.*, 1909, LIX, 1775.

[2] Koller, E. "Zum Vorkommen von 'Herzfehlerzellen' im Harn." *Wiener klin. Wchnschr.*, 1911, XXIV, 636.

large, and at times a large, round nucleus is visible (Bittorf). They are frequently much degenerated.

(2) **Pus.**—In health the urine may contain isolated pus cells, though they are ordinarily missed altogether. An exception is not infrequently met with in women with leukorrheal discharge. The vaginal secretions become mixed with the urine and, as numerous pus cells may be present in the former, they are, of course, found in the examination of the urinary sediment. It is, therefore, necessary in such cases to thoroughly cleanse the external genitals before collecting the specimen or to obtain the urine by means of a catheter. The second procedure is the more accurate method and is to be preferred. The presence in the urine of abnormal numbers of pus cells gives rise to the condition designated *pyuria*. When only a few cells are present there is no macroscopic alteration in the appearance of the urine, but marked pyuria causes a turbidity, and in extreme cases the urine may even appear creamy.

The pus cells (polynuclear neutrophilic leukocytes) retain their characteristic morphology well in acid urine. When the urine becomes strongly alkaline, the pus forms a ropy, tenacious mass, in which the individual cells are swollen, often distorted, and so greatly degenerated that they may be no longer recognizable. However, in weakly alkaline, amphoteric, or weakly acid urines the cells are generally very well preserved, and may even exhibit ameboid activity.

Microscopically, the protoplasm of the cells is finely granular. The majority of the granules are the neutrophilic granules of the cell, though fat droplets may be more or less abundant. In unaltered cells the diameter is about 12 micra—rather smaller than most of the epithelial cells. To determine the nature of the cells beyond question it is

necessary to demonstrate the typical nuclei. This is best accomplished by the addition of 3 per cent. acetic acid; the polymorphous nuclei are then sharply defined. The specimen is examined with the high power dry objective. Staining the sediment may be tried, but is less satisfactory. Carbol-thionin is one of the best stains for this purpose.

In following a patient with pyuria, it may be desirable to count the pus cells in the urine from time to time. For this purpose the twenty-four-hour specimen should be used, and care must be exercised to prevent bacterial ammoniacal fermentation, otherwise the cells become glued together, making a count impossible. The best chemical preservative for this purpose is formalin. Commercial 40 per cent. formalin is added in sufficient quantity to give a solution of 1 to 2 per cent., the preservative being added to each portion of urine as it is collected. Such a procedure is possible only in a hospital, as a rule; when it cannot be carried out, 15 to 20 c. c. of formalin may be placed in the bottle in which the urine is collected. The formalin prevents bacterial growth and at the same time renders the cell nuclei more prominent. Its disadvantage lies in the fact that the cells are clumped together in certain instances. If an ice chest is available, simple refrigeration is to be preferred to any other method of preservation. The urine, if neutral or alkaline, is acidified with acetic acid. The specimen is well stirred to secure a uniform suspension of the cells, and the count is then made directly from it with the hemocytometer, employing the technique used for counting the blood. If the cells are very numerous, it will be found more convenient to use the red pipette.

With the escape of a purulent exudate into the genito-urinary tract, the albumin of the exudate becomes mixed

with the urine, constituting a *false albuminuria*, if the lesion is extrarenal. It is often difficult to interpret findings when a false albuminuria, such as this, is met with. The question arises whether the albumin is derived entirely from the purulent exudate or in part from the kidneys (true renal albuminuria). The presence or absence of casts is of value in determining the latter; instrumental examination may be decisive. Posner [1] has recorded observations which show that, with 80,000 to 100,000 pus cells per cubic millimeter of urine, only about 0.1 per cent. albumin is added to the urine. By comparing the cell count with the quantity of albumin, the source of the latter may be determined.

The following chemical tests for pus may be applied to the urine or to the sediment:

(a) *The Guiac Test.*—Equal parts of hydrogen peroxid and freshly prepared tincture of guiac (see p. 81), when layered over the urine, cause a blue ring to appear at the line of contact in the presence of pus. It may be necessary to wait a few minutes for the color to appear. The color disappears on boiling, unlike that caused by the presence of blood. The test is quite delicate, but is not specific.

(b) *Meyer's* [2] *Guiac Test* (adapted to the urine).—A drop or two of the centrifugalized sediment is transferred to a test tube about two-thirds full of water. The contents of the tube are well mixed and allowed to extract a few minutes, in order to liberate the oxidizing enzyme of the pus cells. The fluid is halved. On one portion freshly prepared tincture of guiac (*without* hydrogen peroxid) is su-

[1] Posner, C. "Ueber Harntrübung." *Deutsche med. Wchnschr.*, 1897, XXIII, 635.

[2] Meyer, E. (a) "Beiträge zur Leukocytenfrage." *München. med. Wchnschr.*, 1903, L, 1489. (b) "Ueber die cytodiagnostische Bedeutung der Guajakreaktion." *Ibid.*, 1904, LI, 1578.

perimposed carefully, and at the line of contact a blue ring appears, which fades in the course of about a half hour. The remaining portion is boiled actively for two to three minutes, then cooled and treated with tincture of guiac in the manner just described. Boiling destroys the ferment, and the test is therefore negative. The test is delicate, and points definitely to the presence of an oxidase; in the urine the only common source of oxidase is pus. If much albumin is present, the reaction may be inhibited.[1]

(3) **Blood.**—Red blood corpuscles are never found in normal, voided urine, excepting the admixtures of blood which occur during menstruation. *Hematuria* is the term used to signify blood in the urine. A small number of blood corpuscles produce no visible change in the appearance of the urine. With larger quantities the translucency of the urine is lost, it becomes "smoky" in appearance on agitating the specimen, and darker in color. A reddish-brown sediment composed largely of red cells may settle out.

The chemical tests for blood are given in connection with hemoglobinuria (p. 80 *et seq.*).

The microscopic examination is made with the high power dry objective. The erythrocytes may be well preserved, and exhibit their characteristic morphology and color. If laking of the cells has occurred, the majority, or all, of the cells appear as "shadows," i. e., the coloring matter has escaped from the red cell, and only the cell membrane remains. To detect the shadows it is essential that the light be cut off as much as possible. In concentrated urine crenation of the red cells, giving rise to thorn-apple forms, is observed.

(4) **Casts.**—The occurrence of casts in the urine, cylin-

[1] Watson, Helen. Personal communication.

druria,[1] is very frequent in disease, and may also be observed in old age and in association with so-called physiological albuminurias. The casts are derived from the renal tubules. They vary greatly in size, the longest measuring in the neighborhood of 1 mm. The thickness of casts is also variable, but in a given cast the width is quite uniform.

(a) *Epithelial casts* are composed of renal epithelial cells in whole or in part. Any cast to which one or more renal epithelial cells are attached may conveniently be designated epithelial (Emerson). The cells are not of equal size, some being large, others smaller. They have a round or oval nucleus, and are usually flat and polygonal. In most instances the protoplasm of the cells is degenerated, showing fat droplets, albumin granules, or, more rarely, myelin droplets. It is unusual to find true epithelial cylinders possessing a distinct lumen. To distinguish between epithelial and pus cells it is necessary to demonstrate the morphology of the cell nuclei by the addition of dilute acetic acid. The cast may be *mixed,* i. e., it may contain both epithelial and pus cells, it may be partly cellular, partly granular, etc.

(b) *Pus casts*, like epithelial casts, consist in whole or in part of pus cells. The cells are generally smaller and rounder than the epithelial cells. Their protoplasm is finely granular, but is subject to the same degenerations as that of epithelial cells. The cells are characterized by their polymorphous nuclei, which are usually visible only after treating the specimen with dilute acetic acid or a dye. At times the cells are so degenerated that the nuclei are no longer demonstrable.

(c) *Blood casts*, when pure, are clots which form in the renal tubules. However, any cast in which one or more

[1] Emerson, C. P. "Cylindruria." *Jour. A. M. A.*, 1906, XLVI, 5; 89.

blood cells are visible is designated a blood cast. At times the red blood corpuscles are not well preserved; shadows of red cells and granules or crystals of hematoidin may be attached to the cast.

(d) *Fatty casts* result from the fatty degeneration of the cells of epithelial casts, or, less commonly, of pus casts. Often the outlines of the original cells are preserved. The casts usually have a yellowish or even brownish tint. The droplets vary considerably in size, some being almost as large as a cell. Ether dissolves the fat droplets, and they may be stained by adding Sudan III or Scharlach R to the preparation. Occasionally fatty acid needles project from the cast.

(e) *Coarsely granular casts* are whitish, yellow, or very dark brown in color, quite opaque, and are covered, either partly or entirely, by coarse granules, as their name indicates. Some of the granules dissolve in ether and stain with osmic acid or other fat stains, while others are albuminous and are soluble in acetic acid. Occasionally granules resembling myelin droplets are observed. Coarsely granular casts are probably derived from epithelial and pus cell casts. All stages in transition may be seen. The casts are often partly waxy.

(f) *Finely granular casts* resemble the coarsely granular, but they are much less opaque and the granules are much finer. Transitions from the coarsely to the finely granular are met with. The granules may cover part or all of the cast. The non-granular portion of the cast may be cellular; more frequently, it is hyalin. Fat droplets are of much less frequent occurrence than in the coarsely granular variety, and myelin droplets are exceptional. The finely granular casts are best seen with low illumination. They are one of the commonest types of cast in disease.

(g) *Hemoglobin casts* are very rare and are always associated with hemoglobinuria. They are covered with dark, granular pigment; less commonly needles of hematoidin are attached to the casts.

(h) *Waxy (colloid or amyloid) casts* are opaque, very refractive, and white or yellowish in color. Their appearance suggests bodies made of paraffin or wax, according to the color of the cast. They are very brittle, and not uncommonly present transverse fissures or cracks. During centrifugalization they may be broken; the fragments are then seen in the sediment. Some waxy casts give the iodin reaction for amyloid when treated with Lugol's solution. It is not unusual to find the cast in the form of a spiral or corkscrew. Cells may be attached to waxy casts, or they may be granular in part. They are supposed to be derived from coarsely granular casts or from hyalin casts which have remained in the renal tubules for some time.

(i) *Hyalin casts* are pale and very slightly refractive. Unless most of the light is cut off, it is impossible to see them; they are almost glassy in their translucency. They may be stained by dilute gentian violet or by Lugol's solution, and are then easily found. They are usually narrow and have rounded ends. In a urine undergoing ammoniacal fermentation they disappear more rapidly than any other kind of cast. Between the glassy hyalin cast and the waxy cast there is a large intermediate group, consisting of casts which are much less opaque than waxy casts, but—though designated hyalin—possessing considerably more density than the glassy type of hyalin cast. Hyalin casts are supposed to represent albumin coagula from the renal tubules or a morbid, coagulable secretion of the renal cells. They are the commonest variety of cast.

Upon any cast granular, amorphous urates may be de-

posited, producing a finely granular appearance. Bacteria in large number, attached to a cast, produce a somewhat similar picture. The uniform outline of the cast is often lost, and the artefact may be recognized as well by microchemical reactions.

(j) *Cylindroids* are casts, one of whose ends tapers to a thread-like filament. They are usually hyalin, though at times granular, and have the same significance as casts.

Under the name *pseudocast* is included anything which may be mistaken for a cast. Scratches on the glass slide most often mislead the beginner. Particles of dust, fibers, etc., may also cause confusion. The morphology of the cast is not duplicated, and experience soon teaches one to differentiate.

(5) **Mucous Threads.**—Mucous threads, sometimes included with cylindroids, with which they have nothing in common, are normally present in the urine. They make up the nubecula. They appear as long, narrow, translucent bands of mucin, of unequal thickness, often twisted or folded like a ribbon, at times branched. Nearly always a few epithelial cells and, perhaps, an occasional pus cell are attached to the threads. Unlike hyalin casts and cylindroids, mucous threads are insoluble in acetic acid. The length of the threads is at times great; a single specimen may extend through several fields of the microscope.

(6) **"Clap Threads."** — Clap threads (*Tripperfäden*) are white or grayish-white, thread-like bodies which are seen floating in the urine, when the specimen is agitated. They are not always gonorrheal in origin, as the name suggests, though in the vast majority of instances associated with a chronic specific urethritis. They are ½ to 1 cm. or more long, and consist of a matrix of mucus, in which epithelial or pus cells or both are embedded. The pus cells

may be so abundant that the thread is quite opaque and yellowish. In the gonorrheal cases it is often possible to demonstrate the presence of the gonococcus.

MICROORGANISMS IN THE URINE

Gonococcus.—The gonococcus is a diplococcus shaped like a biscuit or coffee bean. It is found either within the pus cells or free in the serum. It is Gram-negative. Therefore, the finding of a diplococcus in urethral pus, of characteristic morphology, which decolorizes with Gram's stain, makes it highly probable that the organism is the gonococcus.

To demonstrate the gonococcus the following procedure may be employed:

(1) A smear of the pus is made on a glass slide. It is dried in the air and fixed by passing it through the flame of a Bunsen burner five or six times.

(2) Stain 1 to 3 seconds with anilin water gentian violet. (Avoid overstaining.) (To prepare anilin water gentian violet, 10 parts of anilin oil are thoroughly shaken with 100 parts of water, and, after standing about five minutes, the rather milky emulsion is filtered through a moistened filter paper. The filtrate should contain no large oil droplets. Now add 11 parts of saturated alcoholic solution of gentian violet and 10 parts of absolute alcohol. The solution keeps not longer than eight to ten days.—Schmorl.)

(3) Wash the preparation immediately in tap water and blot it till dry.

(4) Add a drop of immersion oil, and examine the specimen microscopically for diplococci. If none is found after examining three slides carefully, the chances are that diplococci are not the etiological factor in the production of the purulent exudate. If suspicious organisms are seen, it be-

comes necessary to determine whether they are Gram-negative, i. e., whether they are decolorized after treating them with Gram's iodin solution. The further steps are:

(5) Removal of the oil by wiping the specimen with xylol.

(6) The specimen is now covered with Gram's iodin solution, one to two minutes (iodin, 1.0 gm.; potassium iodid, 2.0 gm.; distilled water, 300.0 c. c.), and then, without washing in water, it is transferred to—

(7) Absolute alcohol, in which it is decolorized, until the specimen is colorless or yellowish-gray, excepting the thickest parts of the smear, which may still retain a little blue. Blot dry.

(8) Counterstaining may be performed with Bismarck brown (vesuvin). [The stain is prepared by dissolving 2 gm. of the powdered stain in a mixture composed of 60 c. c. of 96 per cent. alcohol and 40 c. c. of distilled water. The solution is boiled carefully, and, after it has cooled, is filtered. To prevent bacterial growth, a few drops of carbolic acid may be added (Schmorl).] The stain is allowed to act one to two minutes.

(9) Wash in water, dry, and examine.

After the first staining all bacteria and cell nuclei are colored purple by the gentian violet. Decolorization removes the dye from all Gram-negative bacteria and from the cell nuclei. The counterstaining with Bismarck brown then stains the Gram-negative bacteria and nuclei brown, whereas the Gram-positive organisms retain the violet.

Treponema Pallidum (Fig. 9).—Treponema pallidum (*Spirochæta pallida*), the specific parasite of syphilis, may be sought in the serum obtained from specific lesions. The surface of the lesion is first cleansed to remove Spirochæta refringens and other contaminations as much as pos-

sible, and then, if necessary, the lesion is slightly scarified or rubbed with sterile gauze. By pressure or, better still, by suction apparatus a drop of serum usually slightly blood-stained is obtained. Many red corpuscles render the examination difficult and are to be avoided. The serum may be examined in the fresh state with a dark field illuminator, or preparations may be stained.

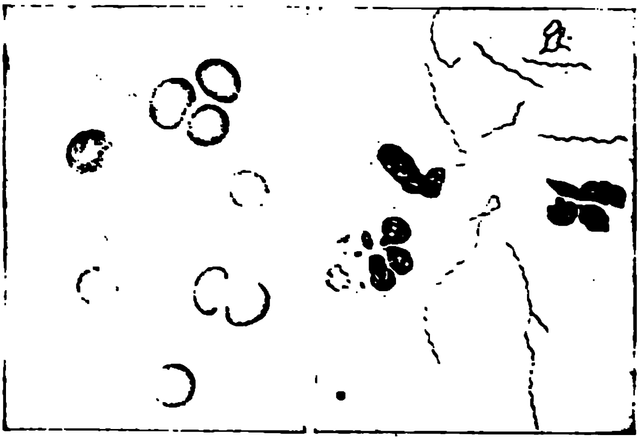


FIG. 9.—TREPONEMA PALLIDUM (Spirochæta pallida) on the left; SPIROCHÆTA REFRINGENS on the right. (After Emerson.)

The Treponema pallidum has a length of 4 to 10 to 20 micra, is very delicate (0.5 micron or less in thickness), has a spiral form, the turns being numerous and close together, and, in the fresh specimen, has a screw-like motion. In spite of its motility its position in the field remains almost stationary. The organism stains very faintly, as its specific name indicates, and is difficult to see.

STAINING METHODS. Smears of the serum are prepared on glass slides or cover glasses, and allowed to dry in the air.

(1) The smears may be fixed and stained with many of the modifications of the Romanowsky stain, such as

Wright's, Leishman's, Wilson's, Hasting's.[1] The technique is the same as that used in staining the blood (p. 285). The Treponema pallidum is usually stained a faint blue, but occasionally has a pinkish color, while Spirochæta refringens is stained a deep blue.

(2) *Giemsa's stain* is also a modification of the Romanowsky stain. It has been employed extensively in searching for the spirochetes. Of the numerous methods of using it, the following are recommended:

(a) The specimens [2] are fixed by immersion in absolute alcohol 15 to 20 minutes or by passing through the flame three times. Ten drops of Giemsa's stain (Grübler's mixture) are then mixed with 10 c. c. of distilled water, shaking after the addition of each drop of stain. (The dilution must be freshly prepared each time the stain is used.) Cover the specimen with the diluted stain, warm it till a slight steam arises, allow it to cool about 15 seconds; the stain is then poured off, and replaced by more of the diluted stain. This procedure is repeated four or five times, when the specimen is washed, dried, and mounted in balsam. The parasites are stained dark red. The slide must be free from grease, and the receptacle for the diluted stain and the staining forceps must be free from acid or precipitated stain. The water used for washing must not be acid.

(b) Giemsa's [3] azure-eosin staining mixture (Grübler's make) is diluted with an equal volume of pure methyl alcohol (Kahlbaum's or Merck's) and placed in a dropping

[1] Geraghty, J. T. "The practical value of the demonstration of Spirochæta pallida in the early diagnosis of syphilis." *Bull. Johns Hopkins Hosp.*, 1908, XIX, 364.

[2] From Mallory, F. B., and Wright, J. H. "Pathological Technique." Philadelphia and London, 5th edition, 1911, p. 418.

[3] Giemsa, G. "Ueber eine neue Schnellfärbung mit meiner Azur-eosinlösung." München. med. Wchnschr., 1910, LVII, 2476.

bottle. It is well to prepare only a small quantity at a time, as it is not known how permanent the solution is. The air-dried films are then placed in a small Petri dish with the specimen side up. The film is now covered with 10 to 15 drops of the alcoholic staining mixture for ½ minute. The preparation is thus fixed and the staining is begun. Add enough distilled water to cover the specimen (usually 10 to 15 c. c.), and agitate the dish till a homogeneous mixture of the stain is secured. Allow the specimen to remain in this mixture 5 minutes. The film is now washed in distilled water, dried, and mounted in balsam. The spirochetes are stained pink.

(3) *Stern's* [1] *Silver Impregnation Method.*—The smears of serum, air-dried, are first placed in the incubator at 37° C. for a few hours. They are then transferred to a colorless glass filled with 10 per cent. aqueous solution of silver nitrate, and exposed to diffuse daylight for several hours. The preparation gradually assumes a brown color. When this has reached a certain shade (quickly learned by practice) and the film shows a metallic luster, it is removed from the silver and washed in distilled water. In a properly treated specimen the spirochetes are stained deep black on a pale brown or colorless background. The organisms are slightly thicker than in specimens stained with Giemsa's stain. Anomalies in staining may be encountered. At times the spirals are more deeply stained at the upper bend of the curve than at the lower, which then appears gray. Or there may be only a row of deep black granules or dots representing a spirochete. The erythrocytes are well preserved, show a delicate, black contour, and present a number of fine granules.

[1] Stern, M. "Ueber den Nachweis der Spirochæta pallida im Austrich mittelst der Silbermethode." *Berlin. klin. Wchnschr.*, 1907, XLIV, 400.

The specimen should not be exposed to direct sunlight while it remains in the silver, for, though the preparation quickly becomes dark and even black, the spirochetes are unstained.

(4) *Burri's India Ink Method.*[1]—One loopful of serum is mixed on a glass slide with a loopful of India ink, and spread in a thin film by means of a second slide. The slides must be perfectly clean. The film, which is allowed to dry in the air, should be dark brown or black. A drop of immersion oil is placed on the specimen, which is ready for examination. Bacteria, spirochetes, blood corpuscles, etc., are unstained, and appear as refractive bodies on the dark background. According to Cohn and others, the best results are obtained with Gunther-Wagner's Chin-Chin black pearl ink, though fair success may be had with other inks, as Carter's or Higgin's.

Bacillus Tuberculosis.—The Bacillus tuberculosis is not easily recognized in the urine because of the constant presence of the smegma bacillus on the genitalia, an organism whose morphology is quite similar to that of the tubercle bacillus, and which cannot be separated from the latter with certainty by staining. It is, therefore, necessary to exclude the smegma bacillus from the urine as a preliminary step in the examination for the tubercle bacillus, as Young and Churchman[2] have shown. The technique which these authors have developed and which has proved reliable is as follows: The foreskin, if present, is rolled back and the glans penis is washed thoroughly with green soap

[1] Cohn, J. S. "On the means of finding the Spirochæta pallida with special reference to the India ink method." *Interstate Med. Jour.*, 1911, XVIII, 26.

[2] Young, H. H., and Churchman, J. W. "The possibility of avoiding confusion by the smegma bacillus in the diagnosis of urinary and genital tuberculosis." *Amer. Jour. Med. Sci.*, 1905, CXXX, 52.

and water, using large amounts of water for the rinsing. The irrigating catheter is now introduced about six inches into the urethra (to the triangular ligament), while the patient keeps the sphincter urethræ closed to prevent fluid entering the bladder. About one quart of sterile water is employed in the irrigation of the urethra. Since the smegma bacillus is not found back of the triangular ligament, the urinary tract is practically freed of this organism by the procedure just described. The patient is now instructed to urinate into three glasses, and a portion of the urine from the third glass is centrifugalized at least five minutes at high speed. Three smears of the sediment obtained are made and stained for tubercle bacilli by the Ziehl-Neelsen method (p. 213). If a thorough examination of the stained specimens reveals no acid-fast bacilli, the result of this particular examination is reported as negative.

Occasionally pus is so abundant that the search for tubercle bacilli is very difficult. When such is the case the antiformin method may be resorted to (p. 214). The pus cells are completely dissolved. It must be remembered that all acid-fast bacteria are resistant to the action of antiformin, so that it is necessary to observe the usual precautions to exclude the smegma bacillus.

ANIMAL PARASITES IN THE URINARY PASSAGES

(1) **Trichomonas Vaginalis.**—Trichomonas vaginalis,[1] a flagellate closely allied to Trichomonas intestinalis, though probably not identical with it, may be found

[1] Dock, G. "Trichomonas as a parasite of man." *Amer. Jour. Med. Sci.*, 1896, CXXXI, 1.

in the vagina and occasionally in the bladder. In either locality it comes in contact with the urine, in which it may be found. It thrives only in an acid medium; this is supplied by the normal vagina, except during the menstrual period, when the mucosa is bathed in the bloody discharge. Trichomonas vaginalis is a pear-shaped organism with pointed extremity and, at the anterior rounded end, presents four flagella. An undulating membrane is also present. It measures usually 0.015 to 0.022 mm. in length and 0.010 to 0.015 mm. in width, though larger and smaller forms occur. It appears to be a specific parasite of the female sex.

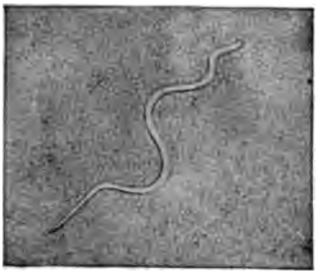


FIG. 10.—EMBRYO OF FILARIA BANCROFTI. × 50. (After Emerson.)

(2) **Filaria Bancrofti.**—Filaria bancrofti (Fig. 10) is of common occurrence in tropical and subtropical countries. Its embryos may be found in the urine in cases of parasitic chyluria. They are either free in the urine and actively motile in a fresh specimen, or caught in the clot. The embryos are 0.125 to 0.3 mm. long, with a thickness of 0.007 to 0.011 mm. (Blanchard). When found or suspected in the urine, the diagnosis should be confirmed by examination of the patient's blood (p. 310).

(3) **Dioctophyme Renale.**—Dioctophyme renale (Eustrongylus gigas), another nematode, is excessively rare in man, though not very uncommon in dogs in this country. It is the largest round-worm parasitic in man. Its habitat is the kidney. Lodging in the pelvis of the kidney, it produces a pressure atrophy until, when the parasite reaches maturity, little or none of the parenchyma of the kidney remains. The male measures 14 to 35 cm. in length, with a

thickness of 0.4 to 0.6 cm. The female is much larger—25 to 100 cm. long and 0.4 to 1.2 cm. thick, and is bright red in color. Infection is diagnosed by finding the ova (Fig. 11) in the urine. The latter are oval, 0.064 to 0.068 mm. in their long axis by 0.042 to 0.044 mm. in the short (Blanchard). The shell is covered with an albuminous coating, which is stained brown and is thrown into ridges, making the surface of the egg appear more uneven than that of Ascaris lumbricoides, which it resembles somewhat.

FIG. 11.—OVA OF DIOCTOPHYME RENALE. ×400. (After Emerson.)

The albuminous coating is lacking at the poles of the ovum and the latter appear colorless.

(4) **Schistosoma Hematobium.**—Schistosoma hematobium (Bilharzia hæmatobia),[1] a trematode, is an important urinary parasite in tropical and subtropical climates. It is especially prevalent in Egypt. The parasite lives in the veins of the urinary bladder; it deposits its ova in the veins. The ova then pass from the veins to the bladder. The ova are similar to those found in the feces (q. v.), except for the fact that the spine is terminal instead of subterminal (Fig. 26). As the sharp-spined ova pierce the wall of the vein, hemorrhage, of course, ensues, with the result that hematuria is a quite constant symptom of the infection.

[1] Lane, C. G. "Bilharziasis; report of a case with appendicitis; literature since 1904." *Boston Med. and Surg. Jour.*, 1911, CLXIII, 937.

PROSTATIC FLUID

Prostatic fluid [1] is obtained by massage of the prostate gland per rectum, the urethra having been irrigated previously. The amount of fluid obtained at a "milking" varies from a few drops to 4 or 5 c. c. The fluid is of low specific gravity, slightly tenacious, grayish-white, yellowish, or greenish in color, and usually has a milky turbidity from the lecithin granules contained in it.

A fresh drop of the fluid is examined microscopically for the presence of motile *spermatozoa*. *Lecithin* granules vary considerably in size. The smallest are minute specks, the largest four micra or more in diameter. They are moderately refractive. *Corpora amylacea*, laminated bodies with a granular center, may be met with, especially in specimens obtained from the aged. They resemble starch granules not only in form, but also in the fact that they may be stained blue with iodin. Various kinds of epithelial cells may be found. In examining for *epithelial and pus cells* it is well to add dilute acetic acid to bring out the cell nuclei. *Spermin crystals* (Böttcher's crystals), transparent needles or whet-stones, are observed at times. They may resemble Charcot-Leyden crystals, but differ from the latter in that they are soluble in alkalies and in formaldehyde.

FUNCTIONAL DIAGNOSIS OF THE KIDNEY

Many tests, some simple, others complicated, have been introduced to measure the functional capacity of the kidneys. All have had certain well-recognized limitations, and

[1] From Emerson, C. P. "Clinical Diagnosis."

none has been particularly helpful where the two kidneys are equally involved in the disease process, as in nephritis, until Rowntree and Geraghty described their phenolsulphonephthalein test. This constitutes by far the most satisfactory and exact method of functional diagnosis, and, in the hands of a number of workers, has proved of immense value in the diagnosis, prognosis, and treatment of both medical and surgical diseases of the kidneys. In surgical affections some of the simpler tests may be used in conjunction with the "phthalein" test. The specimens obtained by ureteral catheterization often permit of accurate diagnostic conclusions through comparison of the microscopic and chemical findings from each kidney. Urea determinations with the hypobromite method are frequently made to advantage; for, though the values obtained represent total nitrogen more nearly than urea, nevertheless the comparative efficiency of the two kidneys may be fairly accurately determined in many instances. The information thus gained is practically always corroborated by the phthalein test, but frequently the latter will give evidence of disease when other tests are misleading. In nephritis and analogous conditions, where each kidney is involved to about the same extent, the phthalein test is the only reliable measure of functional capacity.

The Phthalein Test of Rowntree and Geraghty.[1]—Twenty to 30 minutes before starting the test the patient is given [illegible] of water to insure free urinary secretion. [illegible] with aseptic technique [illegible] of phenol-

[1] [illegible] clinical [illegible]

sulphonephthalein[1] is administered intramuscularly into the lumbar muscles. (The solution is prepared as follows: "0.6 gm. of phenolsulphonephthalein and 0.84 c. c. of $\frac{2}{N}$ sodium hydrate are diluted with 0.75 per cent. sodium chlorid solution up to 100 c. c. This gives the monosodium or acid salt, which is red in color, and which is slightly irritant locally when injected. It is necessary, therefore, to add 0.15 c. c. more of the $\frac{N}{2}$ hydroxid, a quantity sufficient to change the color to a beautiful Bordeaux red. This preparation is non-irritant.")

The catheter is retained until the dye appears in the urine, when it may be withdrawn if there be no obstruction, as from enlargement of the prostate. The urine is collected in a vessel, which contains one drop of 25 per cent. sodium hydrate, since the red color of the drug is apparent only when the reaction of the solution is alkaline. The time of appearance of the dye in the urine is noted. At the end of the first hour after administering the phthalein the patient urinates into a clean receptacle, and into a second receptacle at the end of the second hour. Each specimen is now rendered distinctly alkaline by the addition of 25 per cent. sodium hydrate in order to elicit the maximal color. The dye is yellow or orange in an acid urine, but becomes purplish-red when the reaction is alkaline. Place the urine (each specimen separately) in a volumetric flask of 1,000 c. c. capacity, and add distilled water to 1,000 c. c. Mix thoroughly, and filter a small portion for comparison with the standard solution. When the ureters are catheterized four specimens are obtained in the two hours. Each is examined colorimetrically for phthal-

[1] The substance is supplied by Hynson, Westcott & Co., Charles and Franklin Sts., Baltimore, Md. It is dispensed in glass ampuls. The dose is 1 c. c.

ein content. The functional capacity of each kidney is determined, and the sum of the four determinations indicates the total function.

The standard solution is an aqueous solution of phenolsulphonephthalein containing 6 mg. to the liter, as described above, the solution being rendered strongly alkaline. Colorimetric determination of the quantity of drug excreted in a given specimen is made with the Duboscq colorimeter or with Rowntree and Geraghty's modification of the Autenrieth-Königsberger colorimeter.[1] Employing the Autenrieth-Königsberger instrument (Fig. 12), the standard solution is placed in the wedge-shaped glass. A filtered portion of the urine, rendered alkaline and diluted to one liter, as described, is poured into the rectangular glass. In one side of the case of the instrument there is a narrow slit, the opposite side being frosted glass. With the frosted glass held to the light, the observer looks through the slit and sees the two columns of fluid—urine and standard solution. By means of a thumb-screw, the wedge containing the standard solution is elevated or lowered until the color intensity is alike on the two sides. The percentage of coloring matter in the urine is now read directly from the position of the indicator on the scale.

As a routine procedure Rowntree and Geraghty recommend intramuscular (lumbar) injection of the drug. They employ a Record syringe of 2 c. c. capacity graduated in $\frac{1}{8}$ c. c. "Whereas in the case of phthalein a normal kidney excretes the greater part of the dye injected within two hours of the time of its administration, and then only a small trace for the next two hours, the moderately dis-

[1] This instrument costs about one-fifth as much as the Duboscq colorimeter and is perfectly satisfactory. The Rowntree and Geraghty modification may be had from Hynson, Westcott & Co., Baltimore, Md. It is made by Hellige in Freiburg.

eased kidney secretes a fair amount within the first two hours, say 50 per cent. of that excreted by the normal kidney, but, the concentration in the blood still being high, it continues to excrete a fair amount in the following two

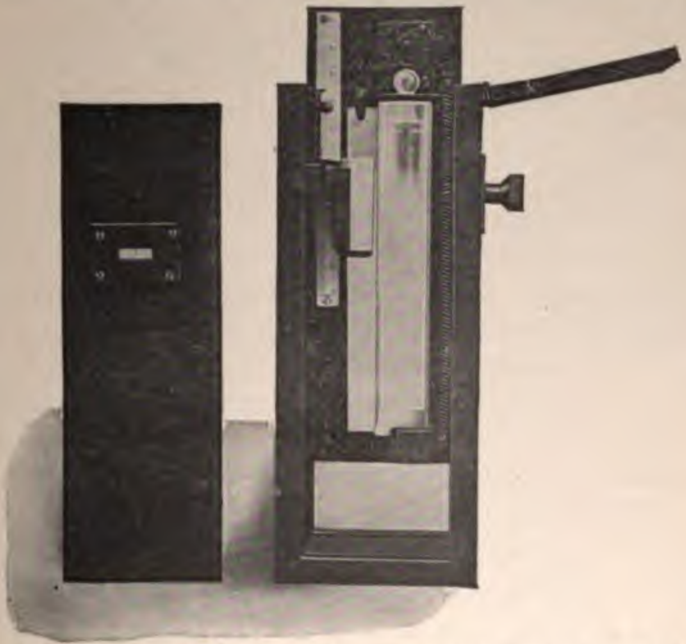


FIG. 12.—THE AUTENRIETH-KÖNIGSBERGER COLORIMETER AS MODIFIED BY ROWNTREE AND GERAGHTY FOR THE DETERMINATION OF PHENOLSULPHONEPHTHALEIN.

hours, so that at the end of four hours little difference may exist in the total work accomplished. One-hour and, at the most, two-hour observations are, therefore, recommended. In cases in which only slight changes in function exist this can be most accurately demonstrated by one-hour collection following the use of an intramuscular (lumbar) injection." With intravenous injection the time of appearance and the duration of maximal elimination are shortened, but the results are, on the whole, less trustworthy.

With *normal* kidneys the following findings have been obtained:

Administration	*Time of Appearance*	*Quantity Excreted*
Intramuscular (lumbar)	5-11 min.	51.8-64.1% first hour
		60-85% two hours
Intravenous	3-5 "	34-45% in 1st 15 min.
		50-65% in 1st 30 min.
		63-80% in 1st 60 min.

As the intensity of color of the dye gradually diminishes in alkaline urine, it is necessary that the determinations be made within a few hours at the most. If the estimation of the dye must be delayed for some hours or days, the urine should be rendered distinctly acid, as the phthalein remains unchanged in acid solution. Just before making the colorimetric determination an excess of alkali is then added to elicit the full strength of the color.

When urine is highly pigmented, error in the colorimetric readings may be lessened by making up a standard solution containing urine. The error from this source is, however, so small as to be negligible in most instances.

CHAPTER II

THE GASTRIC JUICE

The gastric juice is obtained for analysis with the stomach tube, following the administration of a test breakfast or meal. The test breakfasts or meals are employed for the sake of simplicity and to obtain comparable conditions. It is because of the many unknown factors involved, such as the quality of food, the length of time it has remained in the stomach, the condition of the stomach before the food was taken, etc., that little dependence can be placed on the results of analysis of vomitus.

Test Breakfasts.

(1) *Ewald's breakfast* consists of 40 gm. of bread and 400 c. c. of water or weak tea without sugar or cream.

(2) *Dock's breakfast* is the same as the Ewald breakfast, except for the substitution of one shredded wheat biscuit for the bread.

(3) *Boas' breakfast* is prepared by boiling one tablespoonful of oatmeal in 800 c. c. of water till the volume equals about 400 c. c.

In this country Dock's breakfast is rapidly coming into use. This and the Boas breakfast possess a certain advantage over the Ewald breakfast, in that no lactic acid is contained in the food, a possible source of error when bread is used. Any of the breakfasts is allowed to remain in the stomach *one hour*, as a rule, at the end of which the stomach tube is introduced and the gastric contents evacuated.

With normal gastric motility the stomach yields 20 to 50 c. c. one hour after a test breakfast (Boas). To eliminate the possibility of error through giving the breakfast to a patient whose stomach contains part of the previous meal, lavage may precede the breakfast, being performed preferably an hour or so before giving the breakfast. In using the test breakfasts misinterpretation may follow if conclusions are drawn from the results of a single meal.

(4) The *Fischer meal* consists of an Ewald or Dock breakfast with three-quarters of a pound of finely chopped, lean beef, broiled and slightly seasoned. This meal, like the Riegel dinner, excites the secretion of hydrochloric acid better than the breakfasts. It is usually allowed to remain in the stomach three hours.

(5) *The Riegel dinner* is more appetizing than any of the preceding meals. It is composed of:

One plate of meat broth.

Beefsteak weighing 150 to 200 gm. (5 to 7 oz.).

Mashed potatoes, 50 gm. (1½ oz.).

One roll.

Riegel says:[1] "As a rule, I empty the stomach four hours after the meal, provided that other indications are not present that determine me to select some other time. If the stomach is found empty after four hours, I know that the motor power of the organ is good; no conclusions, however, can be drawn in regard to its peptic powers. If the stomach is found empty after four hours, its contents should be withdrawn earlier the next day; if, on the other hand, a large quantity of coarse and only half-digested morsels of food are found after four hours, the examination on the next day should be made later. A single exam-

[1] Riegel, F. "Diseases of the Stomach" (edited by C. G. Stockton). "Nothnagel's Practice." Philadelphia and London, 1905, pp. 79 *et seq.*

ination is never permissible." The dinner is usually given at the time of the midday meal.

Other test meals have been proposed but are not very generally employed for purposes of gastric analysis.[1]

EXAMINATION OF THE FASTING STOMACH

As the examination of the fasting stomach should precede test meals, the results obtained may be considered before passing to the examination of the gastric contents.

The normal stomach empties itself in about seven hours. Passage of the stomach tube before breakfast should, therefore, lead to the recovery of little fluid or none at all. Normally, the amount rarely exceeds 50 c. c. (Emerson). When 100 c. c. or more are obtained, there exists either a gastrosuccorrhea (continuous secretion) or retention of the gastric contents (Boas). Normally or with hypersecretion, swallowed saliva or sputum may be seen in the fluid. With retention, food eaten the previous evening or several days before may be recognized; this should always be looked for, as it furnishes conclusive evidence of stagnation. The ease with which the food may be recognized will depend upon two factors: (1) the chemical composition of the gastric secretion, and (2) the nature of the food. With good acidity proteins may be well digested, whereas with a deficiency of acid they are little altered. Parts of food which resist the action of the gastric juice, such as the seeds of small fruit or berries, are easily detected. In fact, when defective motor power is suspected, it is a useful procedure to give raspberry jam or some similar preparation in the evening, and look for the seeds in the gastric contents or

[1] Riegel, F. "Diseases of the Stomach" (edited by C. G. Stockton). "Nothnagel's Practice." Philadelphia and London, 1905, p. 79 *et seq.*

lavage the following morning. At times excessive quantities of fluid are found in the fasting stomach. The normal organ has a capacity of about 1,600 c. c. (Ewald); a stomach which can retain more than this quantity is dilated.

In addition to the points just enumerated, the fluid obtained from the fasting stomach should be subjected to the examination to be described for the gastric contents.

MACROSCOPIC EXAMINATION OF THE GASTRIC CONTENTS

Quantity.—In the examination of the gastric contents obtained one hour after a test breakfast, the quantity of fluid recovered is measured. Boas finds that the amount usually lies between 20 and 50 c. c. with normal gastric motility. Higher amounts, however, are certainly obtained in health at times; 80 c. c. is not unusual. When 150 to 200 c. c. are found in the stomach, hypomotility is quite definitely indicated. A stomach which is repeatedly found empty one hour after a test breakfast has hypermotility, and it is then necessary to remove the contents after three-quarters or one-half hour.

Odor.—The normal gastric contents are practically odorless. In disease the odor may be sour or rancid (acetic acid, butyric acid, etc.), putrid, fecal, etc. The odor of drugs should also be looked for.

Mucus. The presence of an excess of mucus is most easily detected by pouring the gastric juice from one receptacle to another. If the amount be abnormal, the condition is at once recognized. Mucus from the respiratory passages floats because of the bubbles contained in it. From the pharynx and esophagus there may be a consid-

erable quantity of mucus secreted during the passage of the stomach tube. It runs along the side of the tube, and is not aspirated through the tube, as in the case of true gastric mucus or swallowed sputum.

Color.—Normally the gastric secretion is practically colorless. The regurgitation of *bile* from the duodenum may impart a deep yellow or green color, the intensity depending on the relative proportion of bile. *Blood,* when fresh, is characteristic in appearance; if it has remained in the stomach long enough to undergo change, the bright red color is lost, and is replaced by a dark brown, producing in many instances the so-called "coffee-ground" appearance. The color of the gastric juice may also be altered by food or drugs.

Food.—The state of digestion of the food is of great importance. After the usual test breakfasts, carbohydrate forms the bulk of the food ingested. The alterations found are due chiefly to ptyalin of the saliva. With hyperacidity this enzyme is quickly destroyed, with a consequent inhibition or arrest of amylolysis. After a mixed meal, such as the Riegel dinner, more information may be gained by inspection of the gastric contents. The appearances are well described by Riegel.[1] "In some cases a very fine, uniform, mushy liquid mass is seen that contains no coarse elements at all; in others, again, a mass of food containing many coarse pieces of meat that look as if they had just been swallowed; in addition, there is frequently an abundant admixture of mucus. In some cases there is so much mucus that the food looks like a tough mass and passes through the sound with difficulty, and is very difficult to filter. In other cases there is a large quantity of fluid contents that forms three layers when kept in a glass vessel;

[1] Riegel, F. *Loc. cit.*, p. 86.

at the bottom is seen a layer consisting of fine remnants of amylaceous material; above this a large layer of cloudy fluid, and on the top a foamy layer. If the latter is present it may be considered evidence of gaseous fermentation. This consistency of the stomach contents is found chiefly in cases in which there is stagnation or in which there is motor insufficiency . . . . usually in cases in which there is an abundant quantity of free hydrochloric acid. . . . If the food remnants obtained from the stomach in different diseases are compared, the great significance of macroscopic inspection will be understood. In many instances this method alone will give us diagnostic points which we would otherwise obtain only by complicated chemical examinations. There are cases, for instance, in which the stomach contents do not give any of the reactions for free hydrochloric acid. This shows that there is a deficit in the stomach. Sometimes, however, when free hydrochloric acid is absent, we find only a relatively small amount of finely distributed food residue; at other times we may see larger quantities of coarse food particles. If we limit ourselves to examining the filtrate in both these cases for free hydrochloric acid, we will probably consider that the two are alike, and, as a matter of fact, they are alike in regard to their free hydrochloric acid, for in neither do we see a formation of free hydrochloric acid. If, however, we consider the quantity and the appearance of the stomach contents in both, we shall see that in the first case the peptic power is better than in the second. The first case is functionally nearly normal, for all the albumin has been digested; at the same time there was no residue of free hydrochloric acid. In the second case it is different; here the production of acid was subnormal, as shown by the disturbed digestion of meat.

If this case is more carefully examined, it will be found that the deficit of hydrochloric acid is large, whereas in the first case it is small. In this way macroscopic examination frequently gives us a clear picture of disturbances of function. . . . ''

The careful macroscopic analysis of the gastric contents, it is evident, is of the greatest value.

CHEMICAL EXAMINATION OF THE GASTRIC CONTENTS

Reaction.—The reaction of the gastric contents is tested with litmus paper. It is usually acid. An alkaline or neutral fluid may be obtained.

Hydrochloric Acid

Hydrochloric acid is the most important chemical constituent of the gastric juice from the clinical standpoint. Normally it is present in excess, i. e., a test for free hydrochloric acid is always obtained.

Qualitative Tests for Free Hydrochloric Acid

(1) **Von den Velden's Methyl Violet Test.**—Add a few drops of a saturated aqueous solution of methyl violet to a test tube nearly filled with water. The dilute solution of the dye should be transparent and violet or purple in color. It is divided equally in two test tubes. To the one add an equal quantity or less of gastric juice, to the other an equal volume of water. Free hydrochloric acid is indicated by a change in color from violet to blue, the portion to which water alone is added serving as a control. The test is said to indicate 0.025 per cent. of free hydrochloric acid. Ac-

cording to Riegel, the test is especially valuable, since, when it is positive, it means that there is sufficient free acid for protein digestion.

A second method of performing the test, which is useful when the amount of gastric juice at one's disposal is small, consists in spreading a thin layer of the dilute methyl violet solution in a porcelain plate, and then placing a drop of gastric juice in contact with it. Where the two fluids run together, the violet color is changed to blue in the presence of free acid.

Lactic acid does not interfere with the methyl violet reaction, since it is given only by 0.4 per cent. or stronger solutions, which never occur in the stomach.

(2) **Günzberg's Test.**—This is the standard test for free hydrochloric acid. It is positive only in the presence of a free mineral acid.

Reagent:

Phloroglucin	2.0 gm.
Vanillin	1.0 gm.
Alcohol, absolute	30.0 c. c.

Dissolve and keep in a brown bottle, tightly stoppered. As the reagent does not keep well, it is advisable to make small quantities, so that it may be renewed every few months. It is well to test the reagent from time to time with dilute hydrochloric acid to prove its reliability.

A few drops of the reagent are evaporated to dryness in a porcelain dish by warming gently over a Bunsen burner. A drop of gastric contents is brought in contact with

the yellowish-brown stain left by the reagent, and is evaporated. If free hydrochloric acid is present, an intense red color develops, where the reagent and gastric juice have mixed. Instead of evaporating the reagent and gastric juice separately, equal quantities of the two may be mixed (one or two drops of each) and evaporated, when the color change appears.

The evaporation must be performed with great care. It is easy to burn the reagent by overheating; the test then fails, even though there be an abundance of free acid present. The degree of heat may be tested by touching the bottom of the porcelain dish with the finger. The dish is held in the flame a second, removed, tested; this procedure, repeated at intervals, accomplishes the desired result with a little practice. Blowing on the specimen when it is removed from the flame hastens the evaporation, and at the same time lowers the temperature. A safer method of evaporation is the use of a water bath.

The test is sensitive to free hydrochloric acid in 0.01 per cent. solution. It is specific in the sense that a positive reaction is only obtained with free mineral acid; organic acids do not give the test.

(3) **Tropeolin Test.**—A saturated alcoholic solution of tropeolin 00 is prepared. Three to four drops of this reagent and a like quantity of the gastric juice are spread over the surface of a porcelain dish, and carefully evaporated to dryness. In the presence of free acid the color becomes violet or blue. The test is less sensitive than either of the preceding tests. It is positive with free hydrochloric acid in a dilution of 0.03 per cent. Lactic acid solutions of 0.24 per cent. or stronger give the reaction (Ewald); in the stomach it is doubtful whether lactic acid ever occurs in sufficient concentration to give the test.

In place of the concentrated alcoholic solution of tropeolin 00, Riegel recommends a saturated aqueous solution.

(4) **Congo-paper Test.**—Filter paper is saturated with a concentrated aqueous solution of Congo-red, and allowed to dry. It is then cut into narrow strips. A piece of the paper is moistened with the stomach contents. Free hydrochloric acid turns the paper deep blue; lactic acid produces a much less intense blue. The test is fairly delicate, but with very dilute solutions of hydrochloric acid the color change is very slight and rather difficult to interpret. Lactic acid is never found in sufficient concentration to lead to difficulty, according to Riegel.

(5) **Töpfer's Test.**—One drop of 0.5 per cent. alcoholic solution of dimethylamidoazobenzol is added to a few c. c. of gastric juice. Free hydrochloric acid produces a bright red color. Organic acids also cause a color reaction, but the color is less brilliant—more of a brick red. The reaction is, therefore, not specific, and is the least reliable of the tests.

Of the tests for free hydrochloric acid, the Günzberg test is the most delicate and at the same time the most reliable. It is a good routine test, and in any case should be employed wherever doubt exists.

In certain instances where it is desirable to have information regarding the acid secretion of the stomach, contraindications to the passage of the stomach tube exist. In such case Sahli's desmoid test may be used.

(6) **Sahli's Desmoid Test.**[1]—This is a test for free hydrochloric acid. It is based on the fact that raw catgut is soluble in hydrochloric acid-pepsin, insoluble in pancreatic and intestinal juices.

[1] Boggs, T. R. "Sahli's desmoid reaction in gastric diagnosis." *Bull. Johns Hopkins Hosp.*, 1906, XVII, 313.

Pills of the following formula are prepared:

Methylene blue	0.05 gm.
Iodoform	0.1 gm.
Ext. glycyrrhiz.	q. s.

The pills should not exceed 3 or 4 mm. in diameter. The iodoform may be omitted. The pill is placed in the center of a square of thin rubber dam, such as dentists use. The rubber is stretched and twisted about the pill. The twisted neck is then tied with three turns of *raw* No. 00 catgut, previously soaked in cold water till soft. Now trim the rubber so that a free edge of about 3 mm. width remains beyond the ligature. The cut edges of the rubber must not cohere, inclosing air, for the pill must sink in water, and it must be watertight.

A pill prepared as described is given to the patient with his midday meal, and the urine, collected 5, 7, 18, and 20 hours afterward, is examined for the presence of methylene blue, iodin, or both. In the absence of the greenish color of methylene blue, the urine should be boiled with one-fifth volume of glacial acetic acid. If the chromogen of methylene blue exists in the urine, the color will then appear. Iodin may be looked for with Obermayer's test for indican (p. 27). If methylene blue appears in the urine within twenty hours after the administration of the pill, the test is considered positive.

A positive test shows that there is sufficient free hydrochloric acid secreted in the stomach to permit of digestion of the raw catgut and liberate the pill from its rubber capsule. If the gastric juice fails to digest the catgut, the pill passes into the intestines and is evacuated. The test is, therefore, one for free hydrochloric acid. As it is given with a regular meal, it encounters the optimal conditions

for acid secretion. The test is thus a useful adjuvant to the usual gastric analyses in certain cases of anacidity.

Organic Acids.—When free hydrochloric acid is markedly diminished or entirely lacking, tests for organic acids should be made. With normal hydrochloric acid values, lactic acid fermentation does not occur. The tests for organic acids are described on pages 141-142.

Quantitative Determination of Gastric Acidity

In the quantitative analysis of the gastric juice the amount of free hydrochloric acid and of total acidity and the extent of the hydrochloric acid deficit are of importance clinically. Very little of diagnostic value has resulted from estimation of the loosely combined hydrochloric acid, i. e., hydrochloric acid in protein combination.

Töpfer's Method for Free Hydrochloric Acid.—This is the method generally employed, since it is quickly carried out and is sufficiently accurate for clinical purposes.

A drop of 0.5 per cent. alcoholic solution of dimethylamidoazobenzol is added to 10 c. c.[1] of filtered gastric contents, placed in a porcelain dish or in a beaker resting on a sheet of white paper for a background. The gastric juice should be measured accurately with a pipette. In the presence of free hydrochloric acid, the addition of the drop of indicator produces a brilliant red color in the liquid. From a burette graduated in tenths of a cubic centimeter, tenth normal sodium hydrate is run into the mixture, a few drops at a time, with constant stirring, till the red color entirely disappears. This is the end reaction. The quantity of tenth normal hydrate required to neutralize the acid in 10 c. c. of the gastric contents is then read from the burette.

[1] If the quantity of gastric contents obtained is small the titration is made with 5 c. c., with a corresponding correction in the final calculation.

The result is usually expressed as "acidity per cent.," i. e., the number of cubic centimeters of tenth normal alkali which would be required to neutralize the free acid in 100 c. c. of gastric contents. Since 10 c. c. were taken, the quantity of alkali used, multiplied by 10, gives the desired result. Normally free hydrochloric acid varies between 20 and 40. The amount of hydrochloric acid may be calculated. One c. c. of tenth normal alkali is equivalent to 0.00365 gm. HCl.

If the amount of gastric juice is small, the same sample may be employed for the determination of total acidity. A drop of phenolphthalein is added and the titration continued. The alkali used in neutralizing the free hydrochloric acid must, of course, be included in the total acidity.

Dimethylamidoazobenzol is not the ideal indicator, since it reacts with organic acids and acid salts as well as with mineral acids. The results obtained with it are, therefore, too high; they do not represent absolute values. Nevertheless, the method fulfills all clinical needs, since the error introduced is relatively so small that it does not vitiate the results for diagnostic purposes.

Other Indicators.—In place of dimethylamidoazobenzol Günzberg's reagent and Congo-red are frequently employed as indicators in the titration of free hydrochloric acid.

Günzberg's reagent may be used in several ways. As the titration progresses, a small drop of the gastric juice is removed with the stirring rod from time to time, and placed on the evaporated Günzberg's reagent. The drop is evaporated, and the red color appears at the margin as long as free acid exists. A second procedure consists in the addition of 25 to 30 drops of Günzberg's reagent to the gastric juice, and then at intervals the removal of a minute drop, which is evaporated in the usual manner. The glass

stirring rod itself may be gently warmed till the fluid clinging to it is evaporated; it is then examined for the red color. The disadvantage in these procedures is that a small quantity of the gastric contents is lost with each test for free acid, so that the result is slightly low. Comparative titrations with Günzberg's reagent and dimethylamidoazobenzol will show less free acid, as a rule, when Günzberg's reagent is used; occasionally the values are alike.

Congo-red paper may also serve as the indicator in the titration of free hydrochloric acid. It is very convenient for night work. The tenth normal alkali is added to the gastric juice until a small drop placed on Congo-red paper no longer produces a blue color. As a control the paper should be moistened with distilled water, for the red color becomes somewhat darker when moistened. The results are usually intermediate between those obtained with Günzberg's reagent and those with dimethylamidoazobenzol.

Titration of Total Acidity.—The total acidity comprises free hydrochloric acid, loosely combined hydrochloric acid (i. e., in combination with protein), acid salts, and organic acids, such as lactic, butyric, and aminoacids, when present. Its quantity is determined by titration with tenth normal alkali, using phenolphthalein as the indicator.

With a pipette measure 10 c. c. (or 5 c. c.) of filtered gastric contents into a porcelain dish or Erlenmeyer flask placed on a sheet of white paper, and add one or two drops of 0.5 per cent. alcoholic solution of phenolphthalein as indicator. In an acid medium it is colorless, but it becomes pink as soon as all the acid is neutralized, leaving a slight excess of alkali. Tenth normal sodium hydrate is added from a burette under constant stirring, until the whole mixture takes on a faint pink color, which persists. The number of c. c. of alkali used, multiplied by 10 (or by 20 in

case 5 c. c. of gastric contents were taken), gives the total acidity per cent. Normally this varies between 40 and 60 or 70.

The results obtained are again only approximately correct, being too high as a rule. For diagnostic purposes the method is practicable.

The Hydrochloric Acid Deficit

A deficit in hydrochloric acid occurs whenever the gastric mucosa secretes so small a quantity of hydrochloric acid that there is not merely an absence of free acid, but an excess of bodies capable of binding or uniting with it. Such bodies are chiefly proteins and their end-products, peptids, and the aminoacids. If peptic digestion of the proteins alone occurs, the aminoacids are not concerned in the production of a deficit in hydrochloric acid, since pepsin is unable to carry the hydrolysis of the protein molecule to the aminoacid stage. But, when trypsin is regurgitated into the stomach, or when the proteolytic enzyme of a malignant neoplasm is secreted into the stomach, aminoacids may be abundant in the stomach contents; they may also be the result of bacterial decomposition, though probably not frequently. The presence of aminoacids is of significance in two directions in the quantitative analysis of the gastric contents, as Fischer [1] has pointed out. Pepsin converts the proteins into peptids, which react alkaline toward litmus; when united with hydrochloric acid the reaction is reversed. The hydrolysis of the peptids into their constituent aminoacids alters the conditions. The latter can bind hydrochloric acid and at the same time carboxyl groups are liberated. The result is that the total

[1] Fischer, H. "Zur Kenntnis des carcinomatösen Mageninhaltes." *Deutsch. Archiv f. klin. Med.*, 1908, XCIII, 98.

acidity is increased, while the free hydrochloric diminishes. With an excess of aminoacids it is then necessary to add more or less hydrochloric acid before a reaction for free acid is obtained. Factors which play a less important rôle in the production of an acid deficit are alkalies introduced with the food or secreted, possibly, in disease.

It is unnecessary to remark that only those specimens of gastric juice which fail to react to Günzberg's reagent for free hydrochloric acid are suitable for the determination of a deficit in acid.

The *method* of determining the deficit in free hydrochloric acid is as follows: From a burette add tenth normal hydrochloric acid to 5 or 10 c. c. of the gastric contents with constant stirring, until a test for free hydrochloric acid is obtained. For this purpose the Günzberg test is to be preferred. Dimethylamidoazobenzol is not well adapted to the titration, since organic acids which are often present react with it; Congo-red paper gives more satisfactory results than dimethylamidoazobenzol. The extent of the deficit may be expressed as "deficit per cent.",—the usual way; the number of cubic centimeters of tenth normal hydrochloric acid which would be required for 100 c. c. of gastric contents is calculated. Or the deficit may be expressed in terms of hydrochloric acid, calculated for 100 c. c. of stomach contents.

Organic Acids

Lactic Acid

Of the organic acids which may be present in the stomach contents in disease, lactic acid is the most important and is the only one tested for in the usual routine exam-

ination. It is odorless. Lactic acid is the result of fermentation of the gastric contents. The fermentation occurs only in the absence or very marked decrease of free hydrochloric acid. When many Oppler-Boas bacilli are present in the gastric contents, lactic acid is usually found, though the converse is not true. Lactic acid almost always means stasis of the gastric contents; it is not found in anacidity, where the motor power of the stomach is normal. Quantitative estimation of lactic acid has not been found of value in diagnosis.

Qualitative Tests for Lactic Acid.—(1) UEFFELMANN'S TEST.—To 15 or 20 c. c. of 1 per cent. aqueous carbolic acid in a test tube, 10 per cent. ferric chlorid solution is added till an amethyst color is produced; usually 1 to 2 drops suffice. If necessary, the solution is diluted till it is transparent, and is then divided equally between three tubes. To the first a few drops of the filtered gastric contents are added, to the second a like quantity of distilled water to serve as a control, and to the third the same amount of dilute lactic acid solution for comparison with tube one. A yellowish-green (canary yellow) color denotes lactic acid or its salts. A similar color reaction may also be given by oxalic, citric, and tartaric acids, by alcohol and dextrose, but these substances can usually be excluded after an Ewald or Dock breakfast.

To avoid error from disturbing bodies, it has been recommended to extract the gastric contents with about ten volumes of ether, which is then evaporated; the residue is dissolved in water, to which the test is applied.

(2) STRAUSS' TEST.—To avoid the sources of error in the preceding test, Strauss employs a specially devised separating funnel, which is used to extract the gastric contents. Above the glass stopcock there are two marks which

correspond to 5 c. c. and 25 c. c. The gastric contents are added to the mark 5, and then ether is poured to the mark 25. The two fluids are mixed thoroughly by shaking, and after they have separated the gastric contents are allowed to escape. Distilled water is then added till the ether again rises to the mark 25. After the addition of one drop of 10 per cent. ferric chlorid solution, shake vigorously, and wait for the fluids to separate. In the presence of lactic acid a greenish-yellow color is imparted to the watery layer. The extraction with ether separates the lactic acid from the interfering bodies. If lactic acid is combined with protein, the test may be negative; but the lactic acid may be freed by the addition of dilute hydrochloric acid, until a test for the latter is given with Congo paper. The test now becomes positive.

(3) KELLING'S TEST.—A small portion of the gastric contents is diluted with 10 to 20 volumes of distilled water. A second test tube is filled with the same quantity of water alone. To each tube add one drop of 10 per cent. ferric chlorid. Lactic acid causes a canary-yellow color. Dilutions of 1:10,000 to 1:15,000 may give a positive reaction. The second tube, containing water and ferric chlorid, serves as a control. As in Uffelmann's test, the color is often perceived most easily by looking down into the test tube, which is held on a white background.

Butyric Acid

Butyric acid fermentation may take place in the presence of considerable quantities of free hydrochloric acid. The odor of butyric acid, resembling that of rancid butter, is characteristic. Boiling the gastric contents accentuates the odor; if a piece of moistened blue litmus paper be held

in the mouth of the test tube, the volatile acid reddens it as it escapes during the boiling. Butyric acid also has the peculiar property of separating as a drop of oil on the addition of a small piece of calcium chlorid.

Acetic acid, like butyric acid, may be recognized by its odor if present in sufficient concentration. Acetic acid, after careful neutralization with sodium hydrate, with the formation of sodium acetate, gives a blood-red color on the addition of a drop of ferric chlorid.

Gastric Ferments

Normally pepsin and rennin, or their zymogens, are constituents of the gastric juice. Alterations in the enzymes in disease are much less frequent and less striking than those occurring in the hydrochloric acid. Whenever the latter is present, it is practically always the case that pepsin is also found. With absence of free hydrochloric acid, tests for the enzymes should be made. Quantitative determination of pepsin has not proved to be sufficiently valuable to warrant its inclusion in the usual routine gastric examinations.

Pepsin

Qualitative Test for Pepsin.—Discs of coagulated egg albumin, ca. 1.5 mm. thick and 5 to 10 mm. in diameter, are cut with a cork-borer or goose-quill. They may be preserved in glycerin, but should be washed in water immediately before use to remove the excess of glycerin. A disc of the coagulated albumin is placed in a few c. c. of gastric contents, and, if necessary, dilute hydrochloric acid is added, till Congo paper gives a test for free acid. The material is then placed in an incubator at 37° C. (or in

the vest pocket). In one-half to one hour the albumin should be digested.

Fibrin, usually obtained from ox blood and preserved in glycerin, may be substituted for the coagulated egg albumin.

Quantitative Methods.—Quantitative methods for pepsin may occasionally be desirable. Several have been proposed within the last few years. The results given by each are relative, not absolute, values.

METTE'S METHOD AS MODIFIED BY NIERENSTEIN AND SCHIFF.[1]—Capillary glass tubes, 1 to 2 mm. in diameter and 20 to 30 cm. in length, are filled with egg albumin by suction, the ends plugged with bread crumbs, and the tubes then placed in boiling water for five minutes. They are then sealed with paraffin or sealing wax. Bubbles appear in the albumin, but are no longer seen after three days, when the tubes are ready for use. If the albumin retracts from the wall of the tube, it should not be used for the test.

Method.—One c. c. of filtered gastric contents is diluted with 15 c. c. of twentieth normal hydrochloric acid. With a file or glass scissors, cut off about 2 cm. of the capillary tube, and place two such pieces in the diluted gastric contents. The test tube is then corked and placed in an incubator at 37° C. for twenty-four hours. At the end of this time the tubes are removed and the amount of digestion of albumin in the four ends of the capillary tubes is measured in tenths of a millimeter, a hand lens being useful for this purpose. An average of the four readings is taken. The square of this number represents the number of units of pepsin present in the diluted gastric contents. Multi-

[1] Farr, C. B., and Goodman, E. H. "The clinical value of the quantitative estimation of pepsin, with special reference to the Mette and ricin methods." *Arch. Int. Med.*, 1908, I, 648.

plying this by 16 gives the value for the undiluted specimen.

Since the albumin from different eggs may react differently, and since the length of time the albumin is boiled affects its digestibility, the method can be relied upon only to show rather wide variations in pepsin.

According to Cowie,[1] the tubes need not remain in the incubator twenty-four hours. He finds that the amount of digestion varies directly as the time. He derives the following formula for calculating the digestion:

If A=the amount of egg white digested,
B=the time the tubes remain in the incubator,
C=the required time for the end reaction,
X=the peptic value of the fluid tested, or the estimated value in millimeters,

then it will be found that $X = \frac{A \times C}{B}$

Rennin

Rennin, the enzyme responsible for the coagulation of milk, may exert its characteristic action in the absence of hydrochloric acid. Rennin zymogen, inactive in itself, is converted into rennin by acid. The zymogen is much more resistant to alkalies than rennin.

Qualitative Test for Rennin.—Three to 5 drops of filtered gastric contents are added to 5 to 10 c.c. of raw, amphoteric, or neutral milk. After mixing thoroughly, the fluid is placed in the incubator for 15 to 20 minutes. The presence of rennin is shown by the curdling of the milk, provided its reaction remains neutral or amphoteric. If

[1] Cowie, D. M. "A rapid procedure for the estimation of the peptic value of stomach fluid by means of the Mette method." *The Phys. & Surg.*, Detroit and Ann Arbor, 1904, XXIV, 118.

the reaction has become acid, it is probable that fermentation or "souring" of the milk is the cause of the curdling. Riegel recommends that equal parts of milk and gastric contents be taken. The latter is first neutralized with tenth normal alkali. Curdling should occur within 15 to 30 minutes. Acid reaction of the milk after incubation invalidates the test, as in the preceding instance.

Rennin Zymogen

Riegel gives the following method for detecting rennin zymogen: Ten c. c. of the gastric contents are rendered alkaline with tenth normal sodium hydrate to inactivate rennin. Then add 10 c. c. of fresh, neutral, or amphoteric milk and 3 to 5 c. c. of 1 to 2 per cent. calcium chlorid solution, and place the mixture in the incubator at body temperature. If the zymogen is present, casein is precipitated within a few minutes.

Pathological Enzyme in the Gastric Contents

It has been demonstrated that malignant neoplasms contain a proteolytic enzyme which, unlike pepsin, is capable of splitting proteins into their constituent amino-acids. This fact has recently been utilized by Neubauer and Fischer [1] in devising a test for cancer of the stomach. The test has been used with varying success by a number of observers. The test is certainly positive in many cases of anacidity unassociated with malignant disease of the stomach. The explanation of the occurrence of the positive

[1] Neubauer, O., and Fischer, H. "Ueber das Vorkommen eines peptidspaltenden Fermentes im carcinomatösen Mageninhalt und seine diagnostische Bedeutung." *Deutsch. Archiv f. klin. Med.*, 1909, XCVII, 499.

test in these cases was not clear, until Warfield[1] demonstrated the presence of a peptid-splitting enzyme in normal saliva. If the saliva is acid or comes in contact with an acid fluid (0.05 per cent. plus), the salivary peptid-splitting enzyme is destroyed. Warfield's observations on the saliva have been confirmed by Koelker,[2] and would seem to invalidate the test of Neubauer and Fischer. As the presence of a peptid-splitting enzyme in carcinomata and sarcomata is well established, it seems probable that not all the positive results are attributable to swallowed saliva. The test, therefore, must be interpreted with extreme caution in its present form. That there are still possibilities of altering the technique, so that more reliable results may be obtained with it, must be admitted, and largely for this reason it is included as given by Neubauer and Fischer.

Method.—An Ewald or Dock breakfast is removed after it has remained in the stomach one-half to three-quarters of an hour. The filtered juice is then tested for the presence of bile (and consequently, in all probability, for pancreatic juice) with dilute tincture of iodin by layering it above the fluid (a green line at the juncture of the two fluids denotes bile pigment), and for blood by means of the guiac test. It is obligatory to exclude both pancreatic juice and blood, since each contains an enzyme capable of hydrolizing polypeptids to aminoacids. If pancreatic juice and blood are lacking, the test for the cancerous peptid-splitting ferment is proceeded with. The gastric juice is tested for preformed tryptophan, as described below, and, if none is found, one adds about 10 c. c. of gastric contents

[1] Warfield, L. M. "A peptid-splitting ferment in the saliva." *Bull. Johns Hopkins Hosp.*, 1911, XXII, 150.

[2] Koelker, A. H. "Ueber ein Dipeptid- und Tripeptid-spaltendes Enzym des Speichels." *Zeitschr. f. physiol. Chem.*, 1911, LXXVI, 27.

to 1 c. c. of glycyltryptophan;[1] the mixture is then covered with toluol to prevent bacterial growth, and is placed in the thermostat at 37° C. for twenty-four hours. A portion of the fluid is then removed with a pipette, and placed in a clean test tube. It is tested for tryptophan, the presence of which indicates the existence of a peptid-splitting ferment, in the following manner: Two to 3 c. c. of fluid are acidified with a few drops of 3 per cent. acetic acid. Bromin vapor is allowed to settle in the test tube till a slight brownish tint is visible in the upper part of the tube; the vapor may be introduced better by means of a 10-c. c. pipette armed with a rubber bulb. The contents of the tube are shaken. If a rose color develops free tryptophan is present, and the test is positive. If the rose color fails to appear bromin is added as before. Unless the color appears the procedure is repeated until the fluid in the test tube shows a light yellow color, which indicates an excess of bromin. An early excess of bromin is to be avoided, since the color reaction is then so evanescent that it may entirely escape notice. Instead of bromin vapor, one-tenth saturated aqueous solution of calcium chlorate may be used. The color reaction is the same, and an excess of the reagent is again to be avoided, as the color may be overlooked.

Mucus

Mucus is always present in the gastric juice, though in very small amount under normal conditions. When pres-

[1] Glycyltryptophan is a dipeptid, which has been placed upon the market in small bottles under the name "Fermentdiagnostikum." Each bottle contains enough glycyltryptophan, with toluol added, for one test. It may also be had from the makers, Kalle & Co., Biebrich am Rhein, Germany, in bulk, and this is to be preferred because of the great saving in cost. In using the small bottles gastric contents are added to the line marked on the bottle, which is then stoppered and placed in the incubator.

ent in excess, the characteristic ropy or stringy quality of the fluid is seen on pouring it from one vessel to another. Microscopically, small snail-like masses of mucus may be seen. Mucus usually contains epithelial or pus cells, if the latter are present in the stomach contents; with normal acidity digestion may leave only the nuclei. Mucus from the bronchi, pharynx, or esophagus is often observed in the gastric contents. Bronchial mucus usually contains bubbles, which cause it to float. Microscopic examination reveals alveolar cells, often laden with coal dust, and an abundance of pus cells frequently; an absence of food particles is often noted. The mucus from esophagus and pharynx, which is generally formed in abundance during the passage of the tube, runs out along the side of the tube. It should not be allowed to mix with the material obtained from the stomach.

Anacidity often causes an apparent increase in mucus, even though there is no overproduction, since that which is formed is not normally digested, and, furthermore, it swells to an unusual degree.

MICROSCOPIC EXAMINATION OF THE GASTRIC CONTENTS

Normally one finds, after a test breakfast, only isolated bacteria, a few desquamated epithelial cells, many starch granules, a few fat droplets, possibly a few yeast cells (not budding), an occasional leukocyte at times, and small particles of mucus. The fresh specimen should be employed for examination, which is made with the dry objectives.

Starch granules are conspicuous. When well preserved, laminations are indicated by the concentric lines. If digestion has not proceeded too far, the starch granules are

stained deep blue on the addition of a drop of Lugol's iodin solution.

Fat droplets are recognized by their appearance and staining reactions (see p. 85).

Blood corpuscles, when present in the gastric juice, are often too greatly damaged to be recognized microscopically. With a recent hemorrhage they may, however, present a characteristic appearance, particularly if enough blood has been shed to completely bind the free acid. Chemically, blood is detected more frequently. The guiac or benzidin tests are usually employed as preliminary tests. (For the technique of the chemical tests see the sections on the urine and feces.)

Pus cells, when well preserved, differ in no way from those observed elsewhere. Generally the protoplasm of the cells has been digested, leaving only the naked polymorphous nuclei.

Eosinophilic leukocytes are a rare finding in the gastric contents.[1]

Microörganisms may exist in the stomach in large numbers in disease. Normally their growth is prevented by the free hydrochloric acid and the rapid emptying of the organ.

Yeasts are introduced into the stomach in small number with the food, but they exhibit no sign of germination. In disease, on the other hand, often in the presence of considerable free hydrochloric acid, an active growth of yeasts may be found. Large colonies may be observed. The cells are oval bodies, smaller than a red corpuscle, which have a greenish, glistening appearance when seen with strong illumination. Characteristic budding forms—three or four cells linked together, with a progressive diminution in size

[1] Moačanin, S. "Ueber das Vorkommen von eosinophilen Zellen im Magensafte bei Achylia gastrica." *Wiener klin. Wchnschr.*, 1911, XXIV, 1335.

—are common. They may be distinguished with the low power, and are easily recognized with the high power, dry objectives.

Sarcinæ are found in the form of bales or packages, or as irregular masses of cells. Like yeasts, they are abundant in the stomach only in disease. Two sizes, large and small, are met with, and the significance of each is the same. They are slightly brownish, and can often be found most easily with the low power. They are usually associated with stasis of benign origin.

Oppler-Boas bacilli are capable of producing lactic acid fermentation, and thus it happens that their growth in the stomach practically always results in the simultaneous presence of lactic acid in the stomach contents. The bacilli are characterized by their great size and lack of motility. They are long and have a tendency to grow in chains, which may at times extend across the field of the microscope. To be of importance, the organism must be present in large numbers. It is Gram-positive. It is seen without difficulty with high power, dry objectives in the fresh, unstained preparation. The bacilli are more frequently associated with malignant disease than with other conditions in the stomach.

Trichomonas intestinalis [1] is rarely found in the stomach. Other protozoa—*Balantidium coli, Lamblia intestinalis, Cercomonas hominis*—are very uncommon. (For descriptions of these parasites see pp. 177-179.)

Crystals are of little importance in the stomach. In bile-tinged specimens cholesterin crystals and spheres of leucin have been observed. Triple phosphate, fatty acid, and oxalic acid crystals have been noted.

[1] Cohnheim, P. "Infusorien bei gut- und bösartigen Magenleiden nebst Bemerkungen ueber die sogenannte Infusiorienenteritis." *Deutsche med. Wchnschr.*, 1909, XXXV, 92.

CHAPTER III

THE FECES[1]

In the examination of the feces it is a matter of prime importance that the material be obtained as fresh as possible. If the examination is delayed beyond a few hours at the most, it is quite possible, and, in fact, probable, that erroneous conclusions will be reached. This is particularly the case with regard to animal parasites. It is necessary, for example, to examine for amebæ before the material has cooled; hookworm ova may hatch in the stool, under favorable conditions, in twenty-four hours. Furthermore, bacterial digestion of food rests, such as muscle fibers, may proceed to such a degree within a comparatively short time after the stool has been passed as to lead to a false impression on microscopic inspection. Were it necessary, examples might be multiplied almost indefinitely.

MACROSCOPIC EXAMINATION OF THE FECES

The approximate amount, form, consistence, and color of the stool are noted, and also all recognizable pathological elements, such as parasites, mucus, blood, pus, gallstones, undigested portions of food, etc. A simple inspection suffices for the determination of most of these points.

[1] As reference works in the study of the feces wide recognition has been accorded "Makro- und mikroskopische Diagnostik der menschlichen Exkremente" by M. L. Q. van Ledden Hulsebosch, Berlin, 1899, and to "Die Faezes des Menschen" by Ad. Schmidt and J. Strasburger. Berlin, 1910, 3rd ed. Both are profusely illustrated.

Amount.—The amount of the feces in health varies between about 120 and 250 gm. in twenty-four hours. The frequency of defecation and the quantity of food eaten largely determine the amount passed at any one time.

Form.—The formed or soft stool of the normal individual requires no description. *Scybali* are the small, hard masses of fecal material which have remained in the bowel too long and have become abnormally dry. They are at times coated with mucus, and not infrequently fresh blood may be seen on their surface. The size of a formed stool should be noted; the small movements, about the thickness of a lead pencil, which are seen in starvation or in pathological states of the large intestine, are abnormal. In diarrhea the stools are fluid.

Color.—The color of the stools in health is derived largely from (1) *hydrobilirubin* (*urobilin*), which is reduced bilirubin; though in breast-fed infants' stools unaltered bile pigment is met with. (2) *Food* may alter the color of the intestinal discharges. With a milk diet, the color is light. An unusually dark color results from eating blueberries, etc., and from drinking red wines. Vegetables rich in chlorophyll, such as spinach, may impart a dark green or olive tint. (3) Certain *drugs* have a marked effect on the color of the intestinal contents. After *calomel* a greenish color may be noted; *bismuth* salts may cause a dark brown or even a black color, due to the black crystals of bismuth suboxid. A normally pigmented stool, which becomes dark on exposure to the air, is often attributable to the use of *iron*. Similarly, if *methylene blue* be administered by mouth, oxidation after the stool has been passed may lead to a dark bluish-green color on its surface.

Among the *abnormal coloring matters* of the feces, (4) *blood* is of great importance. The extent to which the color

is altered depends upon the size of the hemorrhage and its source—whether high or low in the gastrointestinal tract. Very small hemorrhages in the stomach or small intestine produce no perceptible change in the appearance of the feces; these are the so-called "occult hemorrhages," which are recognized only by chemical tests. On the other hand, large gastric or duodenal hemorrhages lead to the so-called "tarry" or black stools. A hemorrhage of any considerable size low in the ileum often manifests itself by the passage of very dark red clots or fluid, the hemoglobin showing less alteration than in the preceding instance. With rectal hemorrhages the blood is bright red, often unclotted, and is seen on the surface of the stool, not intimately mixed with it. (5) *"Clay-colored"* stools gain their name from the resemblance to white clay. They may be due to (a) entire absence of bile pigment, *acholic stools,* with the usual increase of fat which accompanies this condition; (b) the reduction of bilirubin by bacteria may be excessive, giving rise to a colorless compound, *leukohydrobilirubin.* In this case the surface of the stool becomes dark after more or less prolonged exposure to the air, and, unlike acholic stools, hydrobilirubin is demonstrable; (c) with very *excessive fat* content, the normal fecal pigment may be so greatly obscured that the stool is clay-colored. (6) The presence of very large quantities of *pus* or of fluid may cover or dilute the normal pigment to such an extent that the specimen appears lighter than usual. Pure pus, such as one sees after the rupture of an abscess into the rectum, needs no description.

Mucus.—Mucus is present in normal feces, but not in sufficient quantity to be observed macroscopically. Long strings or ribbon-like masses of mucus, tenacious and slimy, usually slightly stained with urobilin, may be observed in

disease, or, again, the stools may be encased in mucus, giving an appearance very suggestive of a membrane or a sausage-skin. More frequently smaller particles of mucus are found, varying in size from a split pea up to that of an almond or larger, at times blood-stained or mixed with pus or eosinophilic cells.

Gall-stones.—Gall-stones or other concretions may be found, when present, in the following manner: A bowl or other vessel of about one liter capacity is lined with a double thickness of surgical gauze of sufficient size to permit the free margin to extend well beyond the edge of the bowl on all sides. The stool is now placed on the gauze in the bowl, and the free edges of the gauze are securely tied, so that the stool is contained in a bag of gauze. The specimen is left in the bowl, which is now placed under a stream of running water, where it is allowed to remain until all the finer particles of the feces have been washed away. Gall-stones, unless very minute, cannot pass through the meshes of the gauze, and are, therefore, found in the bag.

This procedure is applicable to the detection of the larger fruit seeds, foreign bodies, etc.

Parasites.—Parasites, such as Ascaris lumbricoides and the larger cestodes, are striking objects which arrest attention at once. Methods for the detection of the smaller worms are described in connection with the hookworm.

INTESTINAL TEST DIET

In the study of functional and anatomic alterations of the intestine, a uniform diet is desirable for many reasons; microscopic and chemical examinations are greatly simplified, and there is supplied a basis of comparison which is

not possible when patients are free to choose their own food.

The test diet of Schmidt and Strasburger[1] is that generally used. Five small meals are given, the first on waking, the fourth in the afternoon.

Diet No. I.—*In the Morning.*—One-half liter of milk, or of tea, or cocoa cooked with milk or water. One roll with butter and 1 soft-boiled egg.

Breakfast.—One dish of oatmeal cooked with milk and strained, with salt or sugar as desired. Instead of oatmeal, gruel or porridge may be taken.

Noon.—One-quarter pound of chopped, lean beef, broiled in butter, rare. A fairly liberal portion of potato purée.

Afternoon.—Same as in the morning, without the egg.

Evening.—One-half liter of milk *or* 1 dish of oatmeal prepared as for breakfast. One roll with butter. One or 2 eggs, soft-boiled or scrambled.

Diet No. II.—For quantitative studies Schmidt and Strasburger[2] recommend a diet containing the following foods, which must be carefully measured: 1.5 liters of milk, 100 gm. of zwieback, 2 eggs, 50 gm. of butter, 125 gm. of beef, 190 gm. of potatoes, oatmeal made from 80 gm. of dry meal, 2 to 3 gm. of salt. They suggest the following arrangement for giving the food:

In the Morning.—0.5 liter milk and 50 gm. zwieback.

In the Forenoon.—Strained oatmeal prepared from 40 gm. of oatmeal, 10 gm. of butter, 200 c. c. of milk, 300 c. c. of water, 1 egg, and salt.

Noon.—125 gm. of chopped beef (raw weight) broiled

[1] Schmidt, A., and Strasburger, J. *Loc. cit.*, pp. 5-6. *Also* Schmidt, A. "The examination of the function of the intestines by means of the test diet, etc." (Translated by C. D. Aaron.) Philadelphia, 1909.

[2] *Loc. cit.*

with 20 gm. of butter; the beef should remain raw on the inside; 250 gm. of potato purée prepared from 190 gm. of mashed potato, 100 c. c. of milk, 10 gm. of butter, and salt.

Afternoon.—Same as in the morning.

Evening.—Same as in the forenoon.

The authors usually give the diet for three days, occasionally longer. To determine when the food has passed through the intestinal tract, they give 0.3 gm. of powdered carmine in capsule with the first meal of the test diet. The carmine produces a red color in the feces.

This second diet, it is calculated, contains 102 gm. of protein, 111 gm. of fat, and 191 gm. of carbohydrate. It is equivalent to 2,234 calories.

Weight of Dried Feces.—The feces are dried on a steam or water bath. The weight of the dried feces of normal adults on Schmidt's diet No. II varies between about 45 and 62 gm.

CHEMICAL EXAMINATION OF THE FECES

For chemical examination a fresh specimen of feces should always be used.

Reaction.—The reaction of normal feces is neutral, faintly alkaline, or faintly acid. It is tested with litmus paper. If the stool is formed or soft, a small portion for testing should be rubbed in a mortar with a little distilled water.

Pigments.—The normal fecal pigment is hydrobilirubin (urobilin). In breast-fed children, however, the bilirubin is not reduced by bacteria, and appears as such in the feces. In disease unaltered bilirubin may be present in the feces; the same is true after active purgation.

UROBILIN (*Hydrobilirubin*)

(1) **Schmidt's Test.**—A portion of the fresh feces the size of a hazel-nut or larger is rubbed in a mortar with three to four times its volume of concentrated watery solution of bichlorid of mercury. The suspension obtained is placed in a covered Petri dish, and set aside for twenty-four hours. All particles stained with hydrobilirubin (urobilin) are colored red, whereas bilirubin becomes green. The color change may appear in less than an hour. The material may be examined microscopically, when even minute particles which contained bilirubin become evident by their green color.

(2) **Schlesinger's Test.**[1]—A portion of the stool is rubbed in a mortar with distilled water to obtain a thin watery suspension. If much fat is present, extract the suspension with ether twice to remove it. Then treat the suspension with acid alcohol (HCl 3 c. c., alcohol to 100 c. c.), and later neutralize the acid with ammonia. Now add to the mixture an equal volume of saturated alcoholic solution of zinc acetate, mix thoroughly, and filter. In the presence of urobilin a green fluorescence is seen.

(3) **Spectroscopic Determination.**—The watery suspension of feces is acidulated with acetic acid, and is then extracted with amyl alcohol. The extract is examined for the bands of urobilin (see p. 71).

BILIRUBIN

(1) **Schmidt's Test.**—This is applied as just described for urobilin. A green color denotes the presence of bilirubin. It permits the recognition of even microscopic bili-

[1] Schlesinger, W. *Loc. cit.* (p. 71).

rubin-stained particles in the presence of an excess of urobilin.

(2) **Gmelin's Test.**—This test is applicable only when a great excess of bilirubin is present (Schmidt and Strasburger). The feces must be examined while fresh. A watery suspension of the material is prepared. Filter paper is soaked in the suspension, and then a drop of yellow nitric acid is placed on the paper. The characteristic play of colors is seen about the edge of the drop—yellow, red, violet, blue, and green, the last at the periphery.

Blood

(1) **Weber's Test.**—A watery suspension of feces is prepared in a mortar. If the stool contains much fat, this is removed by extraction with ether. Then add to the suspension one-third volume of glacial acetic acid and mix thoroughly. If blood is present, the coloring matter is converted into acid hematin. The mixture is now filtered and the filtrate extracted with two to three volumes of ether. Separation of the ether may be hastened by the addition of a few drops of alcohol. Depending upon the quantity of blood present, the ether extract shows a more or less intense shade of brown. The extract is now examined spectroscopically for the bands of acid hematin (see p. 78).

(2) **The Guiac Test.**—If the stool contains too little blood to give the spectroscopic test, about 2 c. c. of the ether extract obtained in Weber's test is treated with about 10 drops of freshly prepared tincture of guiac (a knife-point of powdered guiac dissolved in about 5 c. c. of alcohol), and 20 to 30 drops of hydrogen peroxid or old, ozonized turpentine. The mixture is shaken, and in

the presence of blood a blue color develops throughout the mixture. The color fades after standing a few minutes.

Cowie[1] reports a "water modification" which he finds more delicate. All glassware should be chemically clean and dry. One gram of feces which has been softened with as little water as possible is rubbed in a mortar with 4 to 5 c. c. of glacial acetic acid. To the suspension obtained add 30 c. c. of ether, and shake. To 1 or 2 c. c. of the ether extract add an equal amount of distilled water, and shake thoroughly. Now a knife-point of powdered guiac is placed in the test tube, and is dissolved by agitating the contents. Finally 30 drops of old, water-white, pure turpentine (or hydrogen peroxid) are added, and the contents of the tube mixed. The tube is examined against a white background for the color reaction. If blood is present to the extent of 1 mg. in 1 gm. of feces, a distinct light blue color develops quickly in the ether. With larger amounts of blood the color is, of course, more intense.

Sources of Error.—The guiac test is not a reliable test for blood. It is, however, a very delicate test, and, when it is negative, blood in appreciable quantity is absent. A positive reaction may be given by a great many substances. Of those most apt to lead to difficulty in fecal examinations raw meat, chlorophyll, pus, and salts of the heavy metals are familiar examples. It is advisable to exclude meat and green vegetables from the diet for at least three days before collecting the specimen for examination. All drugs which might interfere with the test, such as preparations of iron, should also be discontinued. A full list of the sub-

[1] Cowie, D. M. "A comparative study of the occult blood tests; a new modification of the guiac reaction; its value in legal medicine." *Amer. Jour. Med. Sci.*, 1907, CXXXIII, 408.

stances reacting with guiac and similar substances—phenolphthalin, aloin, benzidin—is given by Kastle.[1]

The value of the test is concisely stated by Kastle,[1] who says: "The general consensus of opinion among those who have given this subject their attention would seem to be that the guiacum test for blood and similar color reactions are valuable, especially if they lead to negative results, as proving beyond the peradventure of a doubt that blood is absent. On the other hand, if a positive test is obtained, care should be taken to exclude oxidases or peroxidases by boiling, and the salts of the heavy metals and other oxidizing agents by chemical methods, and, if possible, to subject the material under investigation to confirmative tests for blood before finally concluding that blood is present."

(3) **Teichmann's Hemin Crystal Test.**—With a minute particle of dried feces, the hemin crystal test may be performed (see p. 82). With very small amounts of blood the test may fail.

Fat and *starch* are usually recognized without difficulty by microscopic examination. For quantitative determination of neutral fat and fatty acids, works on chemistry should be consulted. (A new method is described by Folin, O., and Wentworth, A. H. A new method for the determination of fat and fatty acids in feces, Jour. Biol. Chem., 1910, vii, 421.)

Enzymes in the Feces

The examination of the feces for enzymes of the pancreas has received considerable attention. Trypsin and amylase (diastase) are most often determined, for their relations in the feces are best understood.

[1] Kastle, J. H. "Chemical tests for blood." *Bull. No. 51*, Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1909, pp. 1-62.

Trypsin

Method of Gross [1] for the Determination of Trypsin.—Reagents:

Solution 1:

Caseinum purissimum (Grübler).	0.5 gm.
Sodium carbonate	1.0 gm.
Distilled water	1,000.0 c. c.

Dissolve by very gentle heating, if necessary. Add toluol to prevent bacterial growth.

Solution 2:

Sodium carbonate	1.0 gm.
Distilled water	1,000.0 c. c.

The feces to be examined are rubbed in a mortar with three times their bulk of solution 2, until a homogeneous suspension is obtained. This is filtered, till the filtrate is clear. Ordinarily the filtration causes no trouble, but if there is much turbidity from bacteria they settle to the bottom, and the clear fluid may be decanted (Gross). Ten c. c. of the fecal filtrate are placed in a flask with 100 c. c. of the casein solution (solution 1). A few c. c. of toluol are added to prevent bacterial decomposition of the casein. The flask is now placed in the incubator at 37° to 40° C. From time to time small portions are removed and tested for casein; this substance is precipitated by dilute (1 per cent.) acetic acid, though the products of its digestion are unaffected. The material is kept in the incubator till the casein has been completely digested. The time is noted.

The rapidity of digestion (and the amount of trypsin)

[1] Gross, O. "Zur Funktionsprüfung des Pankreas." *Deutsche med. Wchnschr.*, 1909, XXXV, 706.

varies with the diet. It is completed most quickly after a protein diet; with carbohydrate food the trypsin is diminished, while intermediate values are obtained with a diet largely of fat. The examination should be made after protein diet; the average time required for complete digestion is 12 to 14 hours, the normal limits being 8 and 15 hours.

Amylase

Wohlgemuth's [1] Method for Determination of Amylase, as Modified by Hawk.[2]—"Weigh accurately about 2 gm. of fresh feces into a mortar (duplicate determinations should be made), add 8 c.c. of a phosphate-chlorid solution (0.1 mol. dihydrogen sodium phosphate and 0.2 mol. disodium hydrogen phosphate per liter of 1 per cent. sodium chlorid), 2 c.c. at a time, rubbing the feces mixture to a homogeneous consistency after each addition of the extraction medium. Permit the mixture to stand at room temperature for a half hour with frequent stirring. We now have a neutral fecal suspension. Transfer this to a graduated 15-c.c. centrifuge tube, being sure to wash the mortar and pestle carefully with the phosphate-chlorid solution, and add all washings to the suspension in the centrifuge tube. The suspension is now made up to the 15-c.c. mark with the phosphate-chlorid solution and centrifugated for a fifteen-minute period, or longer, if necessary, to secure a satisfactory sedimentation. At this point read and record the height of the sediment column. Remove the supernatant

[1] Wohlgemuth, J. (a) "Ueber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments." *Biochem. Ztschr.*, 1908, IX, 1. (b) "Beitrag zur funktionellen Diagnostik des Pankreas." *Berlin. klin. Wchnschr.*, 1910, XLVII, 92.

[2] Hawk, P. B. "A modification of Wohlgmuth's method for the quantitative study of the activity of the pancreatic function." *Arch. Int. Med.*, 1911, VIII, 552.

liquid by means of a bent pipette, transfer it to a 50-c. c. volumetric flask, and dilute it to the 50-c. c. mark with the phosphate-chlorid solution. Mix the fecal extract thoroughly and determine its amylolytic activity. For this purpose a series of six graduated tubes is prepared, containing volumes of the extract ranging from 2.5 to 0.078 c. c. Each of the intermediate tubes in this series will thus contain one-half as much fluid as the preceding tube. Now make the contents of each tube 2.5 c. c. by means of the phosphate-chlorid solution in order to secure a uniform electrolyte concentration. Introduce 5 c. c. of a 1 per cent. soluble starch solution and three drops of toluol into each tube, thoroughly mix the contents by shaking, close the tubes by means of stoppers, and place them in an incubator at 37° C. for twenty-four hours. (In preparing the 1 per cent. starch solution, the weighed starch powder should be dissolved in cold distilled water in a casserole and stirred, until a homogeneous suspension is obtained. The mixture should then be heated with constant stirring until it is clear. This ordinarily takes from eight to ten minutes. A slightly opaque solution is thus obtained, which should be cooled and made up to the proper volume before using.) At the end of this time remove the tubes, fill each to within half an inch of the top with ice water, add one drop of tenth normal iodin solution, thoroughly mix the contents, and examine the tubes carefully with the aid of a strong light. Select the last tube in the series, which shows entire absence of blue color, thus indicating that the starch has been completely transformed into dextrin and sugar, and calculate the amylolytic activity on the basis of this dilution. In case of indecision between two tubes, add an extra drop of the iodin solution and observe them again.

"The amylolytic activity, Df, of a given stool may be

expressed in terms of 1 c. c. of sediment obtained by the centrifugation, as above described. For example, if it is found that 0.31 c. c. of the phosphate-chlorid extract of the stool acting at 38° C. for twenty-four hours completely transformed the starch in 5 c. c. of a 1 per cent. starch solution, then we would have the following proportion:

$$0.31 : 5 :: 1 \text{ (c. c. extract)} : x$$

The value of x in this case is 16.1, which means that 1 c. c. of the fecal extract possesses the power of completely digesting 16.1 c. c. of a 1 per cent. starch solution in twenty-four hours at 38° C.

"Inasmuch as stools vary so greatly as to water content, it is essential to an accurate comparison of stools that such comparison be made on the basis of the solid matter. Supposing, for example, that in the above determination we had 6.2 c. c. of sediment. Since the supernatant fluid was removed and made up to 50 c. c. before testing its amylolytic value, it is evident that 1 c. c. of this sediment is equivalent to 8 c. c. extract. Therefore, in order to derive the amylolytic value of 1 c. c. of sediment, we must multiply the value (16.1) as obtained above for the extract by 8. This yields 128.8 and enables us to express the activity as follows:

$$\text{Df} \frac{38^\circ}{24\text{h}} = 128.8\text{''}$$

This is the method of calculation employed by Wohlgemuth. The departure from the original technique, which Hawk has suggested, consists in the addition of the phosphates to the chlorid solution. The object of this is to secure a uniform medium in which the amylase may be ex-

amined, for it has been shown that the reaction exerts a marked effect on its activity. To do away with this source of error the phosphate-chlorid mixture is employed.

Wohlgemuth finds that the normal average value is about 150.

MICROSCOPIC EXAMINATION OF THE FECES

Unless the stool be very fluid, it is necessary to dilute it with water before examining it microscopically. The method proposed by Stiles is most satisfactory. A drop of water is placed upon a clean glass slide [1] and then, with a flat wooden toothpick or other suitable instrument, a small quantity of feces is transferred to the drop, and mixed with it. During the mixing the slide is inclined and the mixing is done with an upward stroke. By doing so all gritty, solid particles usually are deposited at the upper end, and do not interfere with the spreading of the specimen under the cover glass, which is applied as soon as a thin, uniform suspension of the feces has been secured. The preparation is now ready for examination. Others prefer to examine the preparation without a cover glass. The specimen is of uneven thickness, and has the further disadvantage that parts of it become dry before the examination can be finished.

The specimen should be searched carefully with the low power, and doubtful objects should be examined with a dry lens of higher magnification. Among the objects to be seen in the microscopic examination are remnants of foods,

[1] Large slides, 2 by 3 in., or small glass plates (photographic plates) are convenient for examining feces, as there is less danger of soiling the hands. The toothpick should, of course, be burned or placed in a disinfecting solution immediately after use. *Feces should always be handled as infectious material*, for it is impossible to know when one is dealing with a typhoid or other carrier.

bacteria, granular débris, cells from the mucosa, from blood, or from exudates, parasites, ova, and crystals.

Food Remnants

Muscle fibers are always seen in the feces of patients on a mixed diet. They are yellow in color. As a result of digestion the ends of the fibers are usually rounded, and often only small particles remain. In some of the fibers, however, the striations are well preserved. When digestion of the fibers is faulty, their number is greatly increased, the striations are preserved in the majority, and the ends are square, not rounded.

Fibrous connective tissue may be observed, particularly when there is a lack of free hydrochloric acid in the stomach, since the fibers, digestible in normal gastric juice, are unaffected by the intestinal and pancreatic secretions.

Curds may be seen after a rich milk diet and are frequently encountered in infants' stools. In the latter masses of fat—neutral fat and fatty acids—may bear a close resemblance to curds on macroscopic examination.

Vegetable cells are very varied in shape,[1] and at times are mistaken for parasitic ova by inexperienced workers. The cells are less regular in shape and not so uniform in size as parasitic ova. Measurements are not necessary to demonstrate the great variation in size; it is quite obvious from inspection alone. Often the addition of a drop of Lugol's solution to the specimen will stain the vegetable cells, or at least some of them, blue. If the starch has been digested, this reaction is lost. The larger sheets of vegetable cells cannot be mistaken for anything else.

[1] Hulsebosch (*loc. cit.*) gives excellent illustrations of a great variety of vegetable cells. Schmidt and Strasburger also show many of the commoner cells.

Vegetable spirals are the vessels of plants, which have escaped digestion in the intestine. When tightly coiled they present beaded borders with a latticed appearance between; when drawn out the spiral becomes evident.

Vegetable hairs are not infrequently mistaken for the embryos of parasites. They differ from all known embryos parasitic in man in having a perfectly homogeneous wall, devoid of cellular structure, with a central canal extending throughout. Furthermore, unlike living embryos, they possess no motility. They usually have a yellowish tint and are very refractive.

Starch granules are infrequent in the stools. Their usually oval shape, laminated appearance, and the iodin reaction identify them.

Unrecognizable *débris* is constantly seen in the stools.

Fat in the stools is discussed on p. 171.

Bacteria

Bacteria of all forms are extremely numerous in the stools, except in the case of breast-fed infants. With a few exceptions, little diagnostic importance is placed in their study. In typhoid fever the simplicity and accuracy of blood cultures make fecal examination for Bacillus typhosus superfluous, excepting in the case of typhoid bacillus-carriers with gall-bladder infections. In bacillary dysentery a search for the Shiga bacillus may be desirable; the same is true of Asiatic cholera. (For methods of detecting organisms such as these in the feces, the reader is referred to works on bacteriology.)

Tubercle bacilli are most readily found with the aid of the antiformin method (p. 214). It is necessary to cleanse the anus and surrounding parts in order to remove the

smegma bacilli, as they are normally present in this part of the body. Since tubercle bacilli are frequently present in the feces of patients with pulmonary tuberculosis, due to the habit many adults—and all very young children—have of swallowing the sputa, they do not necessarily indicate an intestinal lesion. It is only when the bacilli are demonstrated in mucopurulent or bloody masses that a diagnosis of intestinal tuberculosis is probable. (For the method of staining tubercle bacilli see p. 213.)

Yeasts, often budding, may grow in the feces, and may be quite numerous. They may be present as a contamination after the stool has been passed.

Sarcinæ and *Oppler-Boas bacilli* may be conspicuous in the feces. Their occurrence in large numbers is probably always secondary to pathological conditions in the stomach favoring their growth. Gram's stain should be used to demonstrate the long bacilli, for other bacteria are so numerous that they are less evident in the fresh specimen than they are in the stomach contents.

Cells

Epithelial cells of the intestinal mucosa desquamate continually. The single small, round, or oval nucleus is usually visible in the cell; if not, 3 per cent. acetic acid should be added. Normally, epithelial cells are few in number. They may be well preserved, but are frequently swollen or otherwise degenerated. They are often found imbedded in mucus.

Blood is never found in normal intestinal contents. It is only when the hemorrhage occurs in the lower part of the intestinal tract that the morphology of the cells is suffi-

ciently well preserved to permit recognition on microscopic examination. Shadows of the red cells may be seen. Clots often contain erythrocytes in a good state of preservation. For the chemical tests for blood see pp. 159-160.

Pus cells—polynuclear neutrophilic leukocytes—in very small number, i. e., an occasional cell, are not pathological. If the cells are not degenerated beyond recognition, the distinguishing feature is the polymorphous nucleus, together with the finely granular protoplasm. The addition of dilute acetic acid may be necessary to demonstrate the nucleus. The cells may be free or embedded in mucus.

Eosinophilic leukocytes are never found in normal feces. They are most frequently, though not always,[1] associated with intestinal parasites or protozoan infections. The cells are often found in particles of mucus, not infrequently blood-stained; Charcot-Leyden crystals are usually found among the eosinophiles.

Crystals

Crystals[2] are commonly seen in the stools. Those of *ammoniomagnesium phosphate* are of frequent occurrence. They are often very imperfect. Crystals of *calcium oxalate* and of *calcium phosphate* are occasionally found. In addition, calcium salts of unknown acids may be precipitated in the feces as irregularly round or oval, bile-stained masses, at times with concentric rings. *Calcium soaps* are constantly present. *Cholesterin* is rarely observed in crystalline form.

[1] Langstein, L. "Zur Kenntnis eosinophiler Darmkrisen im Säuglingsalter." *München. med. Wchnschr.*, 1911, LVIII, 623.

[2] The majority of these crystals have been described in the section on the urine, to which the reader is referred.

Fat.—Needle-like crystals of *fatty acids* and insoluble *soaps* are always encountered on microscopic examination of the feces. The needles are short and slender, and are generally massed, so that the outline of the separate crystals is more or less obscure. At times they are extremely abundant, so much so that they may form the bulk of the stool. Fatty acids are soluble in alcohol and ether; soaps are insoluble. Fatty acids are further differentiated from soaps by the fact that their crystals melt to form droplets on warming. Morphological differentiation between the two may be impossible. Soaps may be present in the form of scales or long needles arranged in clusters. *Neutral fat,* in the form of droplets, may be found in any normal stool. The nature of the droplets may be clear from their varying size, high refractivity, and slightly greenish tint with strong illumination. To identify them with certainty, add to the specimen a drop of Sudan III or Scharlach R (saturated solution in 70 per cent. alcohol), by which the droplets of neutral fat are stained orange to deep orange-red, the intensity of the color depending largely on the size of the droplet.

Charcot-Leyden crystals are never found in normal stools. They are always associated with the presence of eosinophilic leukocytes, though they may persist after the cells have disappeared. They are diamond-shaped, refractive bodies, which may be stained with eosin (see p. 211).

Bismuth suboxid appears in the stools in crystalline form after the administration of bismuth salts by mouth. The crystals are black, irregular rhombs. They are frequently so abundant that the stool is dark or even black in color.

Hematoidin crystals have been observed occasionally.

Intestinal Parasites [1]

In the Southern States and our island possessions intestinal parasites of one sort or another are the rule rather than the exception. Indeed, among the poorer classes of the population practically all are infected in certain localities. The free communication between all parts of the country is constantly disseminating the parasites, so that they are becoming of greater general importance each year. A description is given, therefore, of the important parasites, whose presence may be determined by fecal examination.

The low power objectives are used in the examination of the feces for protozoa, ova, and embryos, the dry objectives of higher magnification being employed for final identification.

Protozoa—Rhizopoda

Entameba Histolytica.—Entameba histolytica [2] (Fig. 13C), found in the stools of those suffering with amebic dysentery, is a protozoan parasite belonging to the class Rhizopoda. When present in the stools, it is most readily found by selecting for examination particles of mucus, especially those which are blood-stained. The mucus may be obtained from the stools after administering a saline cathartic, if necessary, or by passing the rectal tube and re-

[1] The laboratory worker is advised to consult the Bulletins of the U. S. Public Health and Marine Hospital Service, Washington, D. C. Those issuing from the Department of Zoology by Stiles and his co-workers are invaluable to physicians. The excellent book of M. Braun, "The Animal Parasites of Man," translated into English by Falcke, Sambon, and Theobald, is also to be recommended to physicians interested in the parasites of man.

[2] For an excellent discussion of the amebæ of man see Craig, C. F. "The Parasitic Amœbæ of Man." Philadelphia and London, 1911.

moving the mucus which clings to the eye of the tube. In the absence of mucus, the fluid portion of the stool is examined, after giving the patient a saline cathartic. Since the amebæ usually become quiescent soon after the specimen cools, it is absolutely essential that the material be examined at once, while still warm. A warm stage for the microscope is a great advantage, though not a necessity.

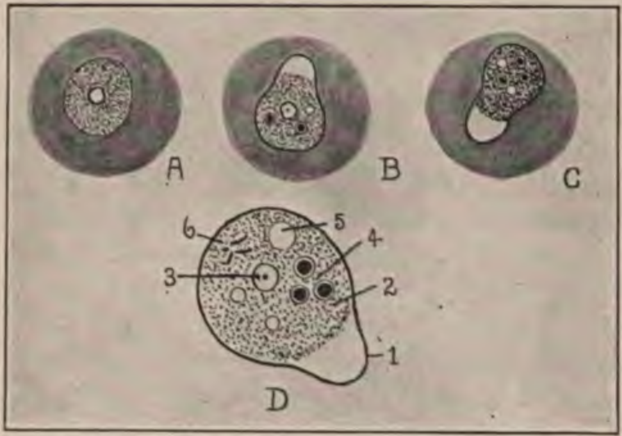


FIG. 13.—PARASITIC AMEBÆ. A, Entameba coli; B, Entameba tetragena; C, Entameba histolytica; D, Diagrammatic; 1, Ectoplasm; 2, Endoplasm; 3, Nucleus, nuclear membrane, centriole; 4, Erythrocytes; 5, Vacuole. (Adapted from Craig.)

In winter, placing the microscope on a radiator often furnishes enough heat to keep the parasites actively motile.

Entameba histolytica measures 0.010 to 0.035 to 0.070 mm. in diameter, though young forms which are smaller may be found. The majority of those seen in the stools are between 15 and 45 micra. The ameboid parasite possesses ectosarc and endosarc, which are well differentiated. The ectosarc has a peculiar greenish color, and, when thrown out to form a pseudopod, it presents an appearance suggesting that of ground glass. It is highly re-

fractive. The endosarc or endoplasm is granular, and contains one to ten or more vacuoles—the younger forms usually only one—which are not contractile. If the intestinal lesions have been bleeding, many engulfed red corpuscles, often in a fair state of preservation, are seen in the endoplasm. The nucleus is not seen, as a rule. The parasite is possessed of ameboid motion which, in a fresh, warm specimen, is very active, rapid, and progressive, the parasite changing its position in a few seconds, so that it may cross the field of the microscope. The ectoplasm is first protruded to form a pseudopod, and then the endoplasm flows into it. A whirling or circular motion of the endoplasm is not infrequently observed. When more sluggish, there may be simply protrusion and retraction of pseudopodia without change of position of the parasite.

The motility of the parasite is necessary for its recognition. In fact, it should be a rule in the diagnosis of amebic infections, to which no exceptions should be made, to refrain from calling any cell an ameba unless actual ameboid motion has been observed in an otherwise characteristic organism.

Simon[1] recommends staining the fresh specimen with dilute neutral red. A drop of dilute aqueous solution of the stain is allowed to run under the cover glass, or a minute particle of the powdered stain may be added to the specimen. There is a selective staining of the endoplasm of the parasite. Ameboid movements seem not to be interfered with. The organisms stand out very prominently.

Stained Preparations.—It is difficult to obtain satisfactory stained specimens of amebæ. They may be stained with hematoxylin or with one of the Romanowsky stains.

[1] Simon, C. E. "Clinical Diagnosis." 7th Ed., p. 214, 1911. Philadelphia and New York.

Brem [1] has had excellent results with a technique of his own. Cover slip preparations are made from the bloody mucus, and the specimens are then stained with Wright's, Hasting's, Leishman's, or Wilson's stain in the following manner:

(1) The unfixed specimen is covered with four drops of the stain, which is allowed to act for 10 to 15 seconds. Since the stain is dissolved in absolute methyl alcohol, this fixes the specimen.

(2) Add to the stain four drops of distilled water. At the end of one minute—

(3) Add four more drops of stain. Again, at the expiration of one minute—

(4) Add four drops of water. The specimen is thus covered with a mixture of stain and water in equal quantities. This is permitted to act for 10 to 30 minutes.

(5) The specimen is now washed in distilled water. (The cover glass should be kept level, while a stream of water is directed against its surface. In this way the precipitated stain is washed or floated off; dumping the staining mixture from the specimen causes the precipitate to adhere to it.) The specimen is quickly dried by holding it over a small flame or by blotting carefully, and is mounted in balsam.

The ectosarc of the amebæ is stained dark blue, the endosarc a light blue. The nucleus takes a brilliant purplish-red color, and bacteria contained in the endosarc have a somewhat similar color. Phagocyted erythrocytes show a pinkish tint.

Entameba Tetragena.—Entameba tetragena (Fig. 13B), which is also a cause of amebic dysentery, resembles both

[1] The method, devised by Dr. Walter Brem of Los Angeles, is unpublished and is given here with his kind permission.

Entameba histolytica and the non-pathogenic Entameba coli. Its diameter varies between 10 and 50 micra, the majority of the parasites being about the size of Entameba histolytica. Like the latter, it has well differentiated ectoplasm and endoplasm. Non-contractile vacuoles are observed in the endoplasm and also erythrocytes, when they are present in the feces. The nucleus of the parasite usually may be seen very distinctly in the endoplasm—a point of resemblance to Entameba coli. The motility of the parasite is quite like that of Entameba histolytica. The organism is said to stain poorly with Wright's stain (Craig). Apparently better results have been obtained with hematoxylin and eosin and other stains (Wetmore).

Entameba Coli.—Entameba coli (Fig. 13A), the non-pathogenic ameba of man, may be found in the feces of 2 to 65 per cent. of healthy individuals after the administration of a saline purgative (Craig). It differs from the pathogenic amebæ in (a) its smaller size, the majority of the parasites measuring 10 to 30 micra in diameter; (b) lack of sharp definition between ectosarc and endosarc; (c) presence of an easily recognizable nucleus, as in tetragena; (d) its opaque grayish color, especially well seen in the younger forms; (e) the small number of vacuoles and absence of erythrocytes in the endosarc (rarely a few red corpuscles may be engulfed when they are present in the feces); (f) the very sluggish ameboid movements with little, if any, change in position in the specimen; and, finally, (g) its reaction to the Romanowsky stains, the ectosarc taking a light blue, the endosarc a dark blue, color—just the reverse of the conditions seen in histolytica.

Resistant encysted forms, by which the infection is transmitted, have been described for the three species of amebæ considered above.

Flagellata

Cercomonas Hominis.—Cercomonas hominis, a flagellate, is, like Trichomonas intestinalis, probably non-pathogenic for man. It is a pear-shaped organism, pointed posteriorly, and measures 0.010 to 0.012 mm. in its long axis (Braun). At the anterior, rounded end a single whip or flagellum about half this length is attached. A nucleus is distinguishable at times near the anterior end. The parasite is actively motile, being propelled by its flagellum. Two or more parasites may become attached to one another by their posterior, pointed ends. The fluid portion of the stool should be examined. The specimen must be examined while perfectly fresh, as the organism dies quickly or assumes a spherical form.

Trichomonas Intestinalis.—Trichomonas intestinalis[1] (Fig. 14) is a pear-shaped body measuring 0.010 to 0.015 mm. long and 0.003 to 0.004 mm. wide (Braun). Freund's measurements are somewhat greater. His smallest organism was 0.009 mm. long and 0.0065 mm. wide, the largest 0.032 mm. long and 0.019 mm. wide. The average of his measurements placed the length at 0.017 mm., the width at 0.010 mm. The posterior end of the parasite is pointed. Four flagella are attached to the anterior, rounded end, and there is an undulating membrane running from the point of insertion of the flagella to the posterior extremity of the organism. The body of the parasite is quite refractive and has a greenish, glass-like

FIG. 14.—TRICHOMONAS INTESTINALIS. ×about 1200. (After Freund.)

[1] Freund, H. "Trichomonas hominis intestinalis; a study of its biology and its pathogenicity." *Arch. Int. Med.*, 1908, I, 28.

appearance in a strong light. Its motility is often so extreme that many of the details of structure of the parasite are seen with difficulty, if at all. The fluid portion of a perfectly fresh stool should be taken for examination. Trichomonas intestinalis is probably non-pathogenic. Encysted forms occur.

Lamblia Intestinalis.—Lamblia intestinalis (Fig. 15), a third non-pathogenic flagellate parasitic in man, appears to be of less frequent occurrence than the two preceding. Viewed ventrally or dorsally, the parasite is pear-shaped.

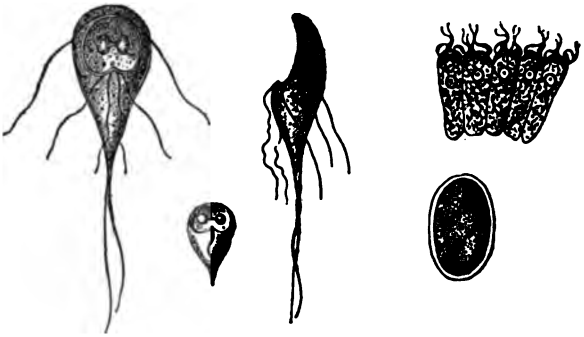


FIG. 15.—LAMBLIA INTESTINALIS. Ventral and lateral views; on the intestinal epithelial cells; and encysted. (After Grassi and Schewiakoff, from Braun.)

It is provided with a cup-like depression or excavation near its anterior extremity, by means of which it attaches itself to the epithelial cells of the mucosa of the small gut. The length of the parasite varies between 0.010 and 0.021 mm., the breadth between 0.005 and 0.012 mm. It is provided with eight flagella 0.009 to 0.014 mm. long, arranged in pairs. The first pair arises from the anterior, the second and third pairs from the posterior, end of the cup-like depression, while the fourth pair is attached to the posterior, pointed extremity of the body. The protoplasm is finely granular. The nucleus may be seen at times beneath the

depression. If intestinal peristalsis is normal or diminished, the forms just described may not be observed in the stools. In their stead encysted forms, which are not characteristic morphologically in the fresh specimen, are evacuated with the feces. Acceleration of peristalsis from whatever cause may lead to the presence of the active stage of the parasite in the stools.

Infusoria

Balantidium Coli.—Balantidium coli (Paramecium coli)[1] is a protozoon which may be pathogenic for man, producing a disease somewhat analogous to amebic dysentery with ulcers in the colon. It is very common in hogs. The parasite (Fig. 16) is oval, 0.060 to 0.100 mm. in its long diameter by 0.050 to 0.070 mm. The anterior end is less pointed than the posterior. A funnel-shaped peristome is situated anteriorly, about which are numerous cilia. Cilia are also conspicuous on the surface of the parasite. Ectosarc and endosarc are visible. The latter is granular and may contain fat droplets, bacteria, mucus, at times erythrocytes and pus corpuscles. Two or more contractile vacuoles are found in the endosarc. A macronucleus, rather kidney-shaped, and a round micronucleus, situated posteriorly, may be seen. Encysted forms, by means of which the infection is transferred, are described.

FIG. 16. — BALANTIDIUM COLI: a, nucleus; b, vacuole; c, peristome; d, food mass. (After Leuckart, from Braun.)

[1] Bowman, F. B. "The pathogenesis of Balantidium coli." *Jour. A. M. A.*, 1911, LVII, 1814.

Dipterous Larvæ.—Larvæ of dipterous insects[1] (flies) may appear in the feces (myiasis). "They are easily recognized. The ringed, cylindrical body, from ½ to 1 inch in length, according to the species, broad at one end, tapering at the other, and usually beset with little spines or hairs, is sufficiently diagnostic" (Manson[2]). It is particularly important that the specimen for examination be fresh for obvious reasons.

Nematodes

Nematodes, round worms, constitute a large class, a number of which are parasitic in man. Their anatomy and biology, though of great interest, are touched upon in the following pages only in so far as they are of diagnostic significance.

Necator Americanus.—Necator americanus (Uncinaria americana), the New World *hookworm,* was first described by Stiles[3] in 1902. Together with its cousin of the Old World, it is by far the most important nematode parasitic in man in this country. It is the causative agent of the disease uncinariasis or anchylostomiasis.

Diagnosis of infection with Necator americanus is made by examination of the stools for the ova of the parasite. The specimen of feces for examination should be *fresh;* in

[1] Gilbert, N. C. "Infection of man by dipterous larvæ, with report of four cases." *Arch. Int. Med.*, 1908, II, 226. (Literature.)

[2] Manson, P. "Tropical Diseases." London, 1900, p. 603.

[3] Stiles, C. W. (a) "A new species of hookworm (Uncinaria americana) parasitic in man." *Amer. Med.*, 1902, III, 523. (b) "The significance of the recent American cases of hookworm disease (uncinariasis or anchylostomiasis) in man." Reprint, 18th ann. rep., Bureau Anim. Industry (1901). Wash., 1902. (c) "Report on the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States." *Bull. no. 10*, Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1903, pp. 1-122.

older specimens—24 to 48 hours—the eggs may have hatched, in which case the embryos, much like those of Strongyloides stercoralis, may be observed. The specimen is examined with low power objective. All doubtful objects are examined with higher magnification. The ovum of the hookworm is characteristic, and, when once seen, can scarcely be mistaken for other bodies in the stool.

The *ova* (Fig. 17) of Necator americanus are oval and possess a clear, colorless shell, which measures 0.064 to 0.076 mm. by 0.036 to 0.040 mm. (Stiles). The outline of the egg is sharp and clearly defined. Inside the shell is the yolk, which is unsegmented when deposited by the female in the intestines, but usually presents two, four, or eight segments or cells, sometimes more, by the time it is evacuated with the feces. The yolk is dark gray or brownish-gray and finely granular; usually a lighter area representing the nucleus may be observed near the center of the yolk cells, especially when their number is eight or less; as the cells multiply, the decrease in size makes this area less conspicuous.

FIG. 17.—OVUM OF NECATOR AMERICANUS. ×460.

In examining a preparation for hookworm (or other) ova, a mechanical stage is a great convenience. About one-half the area of the usual 3x1-in. slide should be covered with the diluted feces, and, before rendering a negative diagnosis, ten such specimens should be examined (Stiles). " . . . To stop after finding a few hookworm eggs is not good practice. The examination should be continued to find, if present, eggs of other parasites, which are likely to be present in small numbers, and to get some idea of the number. When less than ten female worms are pres-

ent, there may be an average of less than one egg to a slide'' (Dock and Bass [1]).

Special Methods.[2]—When the eggs are very few, greater diagnostic accuracy is attained by resorting to special methods for their detection.

(1) *Pepper's Method.*[3]—Pepper has found that hookworm ova possess a peculiar property of sticking to glass. If the preparation for microscopic examination be allowed to settle, immersion in water will remove the greater part of the fecal matter, while the hookworm ova stick to the slide. Dock and Bass find that better results are obtained with the method if part of the fecal material is first removed by centrifugalization.

(2) *Stiles' Method of Washing and Sedimenting.*[4]—''Take one to two ounces of feces, mix with water, and place in a large bottle, retort, jar, or any other receptacle; add enough water to make from a pint to two quarts, according to the amount of feces; shake or stir thoroughly and allow to settle; pour off the floating matter and the water down to near the sediment; repeat the washing and settling several times, or as long as any matter will float. The last time this is done use a bottle or graduate with a smaller diameter, and, when the material is thoroughly settled, examine the fine sediment. It will be found that

[1] Dock, G., and Bass, C. C. ''Hookworm Disease. Etiology, pathology, diagnosis, prognosis, prophylaxis, and treatment.'' St. Louis, 1910. (An excellent discussion of hookworm disease in all its medical aspects, to which the reader is referred.)

[2] Hall, M. C. ''A comparative study of methods of examining feces for evidences of parasitism.'' *Bull. no. 135*, Bureau Anim. Indust., U. S. Dept. of Agric., Wash., 1911.

[3] Pepper, Wm. ''A new method of examination of the feces for the ova of uncinaria.'' *Jour. Med. Research*, 1908, XIII, 75.

[4] *Loc. cit.* (c), p. 85.

the eggs have settled more numerously in the fine sediment than in the coarse material."

(3) *Centrifugalization.*—Simple centrifugalization of the diluted feces often gives disappointing results. The essentials of the method, as given by Dock and Bass,[1] follow: "The feces should be diluted and well mixed with ten or more times their bulk of water. This should be strained through two or three layers of gauze in a funnel to remove the coarse particles. The exact length of time necessary to centrifuge, in order to throw most of the eggs suspended in water to the bottom of the tube, should be determined by experimenting with a known specimen that has already been washed once or twice and contains many eggs. This must be determined with the particular centrifuge used. . . . As the first diluted feces are much thicker than the washed feces and eggs on which the working time of the centrifuge has been determined, the eggs will go down somewhat slower the first time. It is, therefore, a good plan to centrifuge double time at first. If, for example, the working time of the centrifuge is four seconds, the first centrifuging should be eight seconds. This throws to the bottom most things heavier than eggs—like crystals, sand, large vegetable cells, etc.—and all eggs present. There remain suspended in the supernatant fluid nearly all bacteria and fine particles, and many coarse particles lighter and more irregularly shaped than eggs. If the centrifuge is run longer, many of these go down, which is, of course, undesirable. Pour off this fluid and two-thirds, and often more, of the feces are removed by this washing. Refill the tube to about three-fourths its capacity, shake up thoroughly, and centrifuge again, running now only the working time of the centrifuge. It is important not to centri-

[1] *Loc. cit.*, pp. 172 *et seq.*

fuge longer than the working time of the centrifuge, as many fine and light particles would otherwise be thrown down. Considerable material remains suspended and may be removed by pouring off the supernatant fluid. Again the tube is filled, shaken, and centrifuged a proper length of time, and generally this will be sufficient for practical purposes. A part or all of the sediment is removed with a pipette, spread out on a slide, and examined for eggs. It consists of crystals, sand, and heavy, coarse food particles, and eggs, if present. . . . Great care must be exercised to clean the centrifuge tubes before using them after they have had eggs in them. A proper centrifuge brush is serviceable. The method . . . permits the finding of eggs when less than half a dozen laying females are present, and often when only one is present. It is of additional service because it permits at the same time diagnosis of infections with many other worms by which fewer eggs are laid, such as the tenias, oxyuris, bothriocephalus, etc.''

The *adult worms* of Necator americanus (Fig. 18) are found in the stools only after treatment. They are small, whitish, grayish, or reddish-brown in color, and have the anterior end curved dorsally to form a hook. The males measure 6 to 10 mm. in length, the females 8 to 15 mm. The stools, collected after treatment, are placed in a bucket or other suitable receptacle, stirred with several times their volume of water, and allowed to settle a few minutes, when the supernatant fluid is poured off. The washing is repeated several times, and, finally, the sediment is transferred to a plate, preferably with a black background, and the worms looked for. They may be identified by examination under the microscope.

Ankylostoma Duodenale.—Ankylostoma duodenale, the

Old World hookworm, is of frequent occurrence in this country, often associated with Necator americanus. The methods of diagnosis are those described for Necator americanus. The *ova* (Fig. 17) are identical in appearance.

FIG. 18.—NECATOR AMERICANUS. Upper half males, lower half females. Inch measure. (After Dock and Bass.)

Measurements show, however, that they are a little smaller than those of the New World variety, measuring 0.052 to 0.061 by 0.032 to 0.038 mm. The differences are so slight that simple microscopic inspection does not suffice for the separation of the two. The *adult parasites* are a trifle larger than Necator americanus. The males are 8 to 11 mm. long and 0.45 mm. wide; the females 10 to 18 mm. long

and 0.6 mm. wide. There are certain well marked peculiarities by which the two species of hookworm may be differentiated.

Strongyloides Stercoralis.—Strongyloides stercoralis (*Strongyloides intestinalis*), the parasite of Cochin China diarrhea, first reported in this country by Strong[1] and Thayer,[2] has proved to be widely spread through the South-

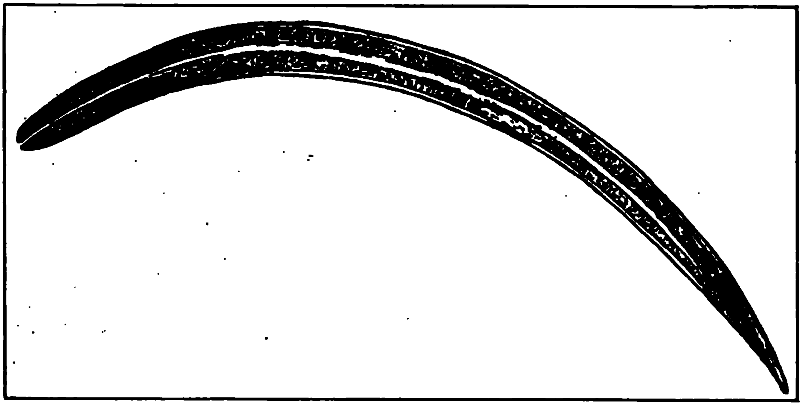


FIG. 19.—THE RHABDITIFORM EMBRYO OF STRONGYLOIDES STERCORALIS. ×460.

ern States, as Thayer predicted. Diagnosis of infection is made by the finding of the actively motile *rhabditiform embryos* in the stool.

Perfectly fresh feces should always be used for examination. If the specimen is kept too long, the embryos may die or may change into the filariform larvæ. If the stool is formed, fluid about it may contain the embryos, or they may be looked for in a preparation of the diluted feces. Since this often fails, even with heavy infections,

[1] Strong, R. P. "Cases of infection with Strongyloides intestinalis (first reported occurrence in North America)." *Johns Hopkins Hosp. Rep.*, 1902, X, 91.

[2] Thayer, W. S. "On the occurrence of Strongyloides intestinalis in the United States." *Jour. Exp. Med.*, 1901, VI, 75.

it is better practice to give a saline cathartic, if necessary, and examine the fluid stools. The embryos (Fig. 19) are 0.450 to 0.600 mm. long and 0.016 to 0.020 mm. thick (Blanchard), and have a characteristic wriggling or squirming motion in the fresh stool. They are grayish-white and quite refractive. They possess a rhabditiform or bottle-shaped esophagus. Embryos which have died are, of course, less conspicuous, but, if well preserved, are characteristic. If the infected stool be kept for one to two days under suitable conditions of light, moisture, temperature, and oxygen supply, the rhabditiform embryos may develop into the filariform larvæ, the infecting stage of the parasite.

FIG. 20.—OVUM OF STRONGYLOIDES STERCORALIS. (Drawn with Leitz obj. no. 7, ocular no. 3.) (After Thayer.)

Ova of Strongyloides stercoralis (Fig. 20) are extremely rare in the feces. They resemble the ova of the hookworm. In one of his cases Thayer found two eggs on daily examination of the stools for several months.

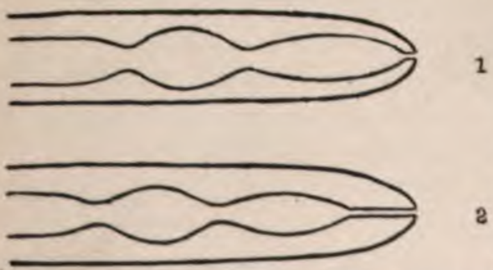


FIG. 21.—THE RHABDITIFORM EMBRYO OF STRONGYLOIDES STERCORALIS (1) AND THE EMBRYO OF THE HOOKWORM (2). Showing the difference in length of the buccal capsule. Diagrammatic.

Hookworm embryos which have developed in the stools (twenty-four to forty-eight hours after passage) may be mistaken for the embryos of Strongyloides stercoralis; the former never occur in the freshly evacuated feces. They are most readily differentiated by the fact that the buccal capsule (Fig. 21) is very short in the embryo of

Strongyloides stercoralis, relatively long in the hookworm embryo (Stiles). In case of doubt, a perfectly fresh stool should be obtained after a saline cathartic, when ova without embryos are found with hookworm infection alone; with double infection, both embryos and ova are seen.

Students often confuse plant or vegetable hairs with (dead or inactive) embryos. The former are distinguished by a straight central canal with hyalin, refractive walls of quite uniform thickness and devoid of finer structure.

The adult parasite, which inhabits the small intestine, is probably a parthenogenetic female, and is a rarity in the stool. It is quite minute—2.2 mm. long and about 0.034 mm. thick.

Oxyuris Vermicularis.—Oxyuris vermicularis (*Ascaris vermicularis*), the common pinworm or seatworm, is a small nematode, the males measuring 3 to 5 mm. long with the posterior end curved, the females about 10 mm. long and 0.6 mm. thick. The *ova* (Fig. 22) are flattened on one side, measure 0.050 by 0.016 to 0.20 mm. (Braun), and have a clear, thin shell. The ova are deposited with the embryos already developed within the shell. It is necessary to recall the fact that the gravid females habitually wander from the region of the cecum and appendix to the rectum, anus, perineum, etc., in order to appreciate clearly the reasons for the methods of diagnosing infection. The crawling of the worm over the skin causes itching, so that the patient usually scratches the affected part.

Fig. 22.—[illegible]

The presence of pinworms may be determined[1] by (1) examining the feces for the adult worms (p. 184). Usually [illegible] are found. The material for examination

[1] [illegible] "Modern Medicine," Vol. I, 1907, p. 601.

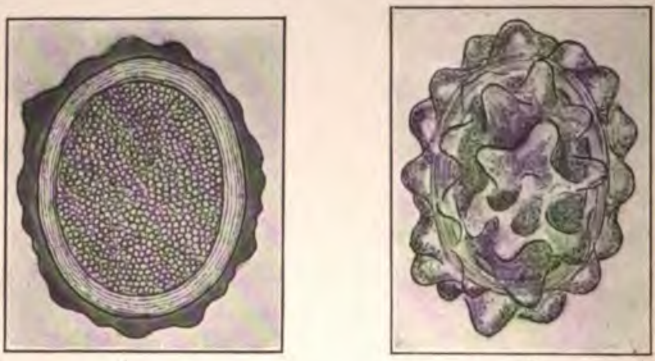
is best obtained by an enema given in the evening. (2) The worms may be seen in the crotch, especially if the child be examined during the restless period after retiring. (3) Microscopic examination of the scrapings of the skin about the anus or of dirt from the finger nails (the ova being picked up in scratching the perineum) may reveal the ova. (4) Eggs may be found in the feces. Fecal examination for the eggs of the parasite is the least trustworthy of the methods of diagnosing infection. Stiles' statement, which is agreed to by experienced observers, should be borne in mind. He says: "The writer's experience is that the eggs may be found in fecal examination in some cases in which pinworm is not even suspected; but that a negative examination is not of much value." Examination for the mature worms or for the ova in the scrapings of the perineum or finger nails give more reliable results.

Trichuris Trichiura.—Trichuris trichiura (*Trichocephalus dispar*), the whipworm, is rarely seen in the feces. It may be found after treatment has been administered. The males are 35 to 45 mm. long, the females 35 to 50 mm., three-fifths of which is formed by the anterior filamentous portion (Blanchard). Diagnosis of infection is made by finding the ova in the feces. The *eggs* (Fig. 23) are oval in shape, with a relatively thick shell, which is generally stained dark yellowish-brown. At either pole there is a space in the shell, which is occluded by a plug, the outer surface of which projects slightly beyond the shell. Within the shell the yellowish or brownish granular yolk substance is seen. The dimensions of the eggs are 0.050 to 0.056 mm. long and 0.024 mm. wide (Blanchard). Though smaller than many

Fig. 23.—Ovum of Trichuris trichiura. ×460.

other eggs, they are, nevertheless, of sufficient size to be easily seen with the usual low magnifications.

Ascaris Lumbricoides.—Ascaris lumbricoides, the ordinary "roundworm" of man, is the largest of the commoner parasitic nematodes. Diagnosis may be made by the discovery of the parasite in the feces or vomitus, or of its ova in the stool. The living worm has a reddish or grayish-yellow color. The males vary in length between



1 2

FIG. 24.—OVUM OF ASCARIS LUMBRICOIDES (1); THE SAME UNDER HIGH FOCUS, SHOWING THE ALBUMINOUS COATING (2). ×460.

15 and 25 cm., and are about 3 mm. thick. The posterior end is conical, and is curved ventrally. Females are 20 to 40 cm. long and about 5 mm. in thickness. Lateral, dorsal, and ventral stripes run longitudinally along the body of the parasite, the first being the most prominent. The *ova* (Fig. 24, 1 and 2) are elliptical and have a thick, transparent shell, which at times appears laminated. A rough albuminous coating forms the outer surface of the egg, and is usually stained brown with the fecal pigments. The albuminous coating may be lost in some of the eggs. The size of the ova varies between 0.040 to 0.050 by 0.050 to

0.070 mm. (Braun). *Unfertilized ova*[1] (Fig. 25) may be encountered. They are flatter—much less plump than the fertilized specimens—the shell is thinner, and the albuminous coating appears to be less abundant. The yolk is coarsely granular, in contrast to the finely granular appearance in the fertilized eggs.

Toxocara Canis.—Toxocara canis (*Ascaris mystax*), the common roundworm of dogs and cats, is rare in man.[2] The adult parasite is much smaller than Ascaris lumbricoides; the males are 4 to 6 cm. long and 1 mm. thick, while the females measure 6 to 11 cm. in length and 1.7 mm. in thickness. The maximal length recorded is 20 cm. (Blanchard). The *ova* resemble those of Ascaris lumbricoides, but are more spherical, having a diameter of 0.068 to 0.072 mm. (Blanchard).

FIG. 25.—UNFERTILIZED OVUM OF ASCARIS LUMBRICOIDES. ×460.

Trichinella spiralis.—The adult males of Trichinella spiralis are 1.4 to 1.6 mm. long and 0.04 mm. thick; the females are larger, measuring 3 to 4 mm. in length, with an average thickness of 0.06 mm. (Blanchard). They inhabit the small intestine. The feces, obtained by active purgation, are mixed thoroughly with water, placed in a tall cylinder, and after the sediment has settled the fluid is poured off. The sediment is then placed in a dish with dark background; the thickness of the fecal layer should not exceed 1/12 inch. The dish is tilted, and any minute, hair-like objects are transferred to a slide and examined microscopically (Stiles).

[1] Logan, O. T. "The little known atypical (unfertilized) egg of Ascaris lumbricoides." *N. Y. Med. Jour.*, 1907, LXXXVI, 1164.

[2] Biesele. "Ueber einen Fall von Ascaris mystax beim Menschen." *München. med. Wchnschr.*, 1911, LVIII, 2391.

The females may remain in the intestines eight weeks; the males die within a few days. The embryos may be recovered from the blood (p. 310).

Trematodes

Trematodes [1] or flukeworms are fortunately rare in the United States, the cases reported being largely importations from Asia and Africa. Of those whose presence in the body may be determined by finding the ova in the feces, the following are among the more important. The parasites themselves are rarely seen during the life of the host.

Opisthorchis Sinensis.—Opisthorchis sinensis, a liver-fluke, deposits dark brown, oval eggs with sharply defined operculum or cap. They gain access to the bowel by way of the biliary passages. The ova measure [2] 0.015 to 0.017 by 0.027 to 0.030 mm.

Fasciola Hepatica.—Fasciola hepatica, also a liver-fluke, is common in many domestic animals (chiefly herbivora), though rare in man. The eggs are oval, yellowish-brown, with distinct operculum, and measure 0.130 to 0.145 by 0.070 to 0.090 mm. They contain no embryo when oviposited.

Schistosoma hæmatobium, the causative agent in bilharziasis (venous distomatosis), inhabits the branches of the portal vein of man, particularly the mesenteric veins, and also the veins of the urinary bladder and vagina. The sharp-spined ova pierce the wall of the vessel, and thus it happens that they may be found in either urine (see p.

[1] Stiles, C. W. "Illustrated key to the trematode parasites of man." *Bull. no. 17,* U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1904. (Illustrations and full descriptions of parasites and ova are given, together with brief clinical notes, keys to the ova, etc.)

[2] The measurements of all trematode ova, unless otherwise indicated, are taken from Stiles (*loc. cit.*) and are the extremes reported by him from the literature or his own observations.

118) or feces, or in both. The eggs (Fig. 26) are oval or spindle-shaped and measure 0.050 to 0.073 by 0.120 to 0.190 mm. (Braun). The shell is clear, usually brown in color, and is provided with a sharp spine. The latter is usually situated laterally (subterminally) near one pole of the ovum when seen in the feces, whereas a terminal spine is commonly seen in the urine. The ovum contains a ciliated embryo or miracidium. At times the latter may be seen swimming free in the preparation. The stool contains blood practically without exception.

FIG. 26.—OVUM OF SCHISTOSOMA HÆMATOBIUM. (From a specimen preserved with formalin.) ×460.

Schistosoma Japonicum.—Schistosoma japonicum is endemic in Japan and in certain parts of China, and has been reported from the Philippines. It inhabits the portal and mesenteric veins chiefly; the bladder is apparently unaffected. Infection of the lung (ova in sputum) is rare. The ova (Fig. 27) are without spine or operculum, oval in shape, and measure 0.060 to 0.090 by 0.030 to 0.050 mm. (Braun). Each contains a fully developed miracidium.

FIG. 27.—OVUM OF SCHISTOSOMA JAPONICUM. (From a specimen preserved with formalin, obtained through the kindness of Dr. O. T. Logan.) ×460.

Fasciolopsis Buskii.—Fasciolopsis buskii, like Schistosoma japonicum, is

an intestinal fluke. It is widespread in Asia. The ova measure 0.120 to 0.130 by 0.077 to 0.080 mm. and have a delicate operculum.

Paragonimus Westermanii.—Paragonimus westermanii, the lung-fluke, belongs to the class of parasites under consideration. Its ova may appear in the feces through swallowing of the sputa, if they pass the stomach intact. Liver infection has also been recorded. The eggs (Fig. 36) are oval, 0.068 to 0.118 by 0.048 to 0.060 mm. in size, possess a yellow shell, and are provided with an operculum.

Cestodes

Cestodes [1] or tapeworms include some of the commonest intestinal parasites in the United States. The larger worms usually disclose their presence to the infected individual by segments, which appear in the feces. Microscopic examination of the stools oftentimes reveals ova where infection has not been suspected.

Tenia Saginata.—Tenia saginata, the beef tapeworm, is a large parasite, measuring 4 to 10, even 36 m. in length when fully developed. From the mature, gravid segments (the segments are hermaphroditic), *ova* (Fig. 28, 2) are deposited in the feces. They are round or oval, and measure 0.030 to 0.040 by 0.020 to 0.030 mm. (Braun). The shell is rather thick, radially striated, and light brown in color. Within it three pairs of hooklets may be visible; to see them it is necessary to focus carefully, as it does not happen often that all are in the same plane. The mature *segments* or *proglottides* (Fig. 28, 1) are those usually seen in the feces. They are 16 to 20 mm. long and 4 to 7 mm. broad, and are characterized by the presence of a uterus

[1] Stiles, C. W. "Illustrated key to the cestode parasites of man." *Bull. No. 25*, Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1906.

with central stem, from each side of which 25 to 30 lateral branches are given off; these lateral branches are themselves subdivided into numerous smaller branches. The gross structure of the uterus may be determined by flattening the segment between two glass slides and holding

(1) (2) (3)

FIG. 28.—(1) GRAVID PROGLOTTIS OF TENIA SAGINATA (×4); (2) OVUM OF TENIA SAGINATA (×460); (3) GRAVID PROGLOTTIS OF TENIA SOLIUM (×4).

it to the light. The uterus then stands out in fairly sharp relief. Each segment is provided with a genital pore which is found at one side; the pores alternate very irregularly from side to side. The head of the parasite is cuboidal, 1.5 to 2.0 mm. thick. It is unarmed.

Note.—From the ova alone it is impossible to distinguish between Tenia saginata and Tenia solium (q. v.). The ova of Tenia saginata are quite innocuous to man, since the intermediary stage of the parasite, Cysticercus bovis, to which they give rise, develops practically only in beef—at all events, not in man. With Tænia solium the case is quite different. While the hog is the usual host of

the Cysticercus cellulosæ, the latter may also occur in man, either from the introduction of the ova or the mature, gravid segments into the stomach. Obviously, then, it is very important to handle all intestinal discharges containing ova like those described above with extreme care, until the presence of Tenia solium is definitely excluded. For similar reasons, the patient's excreta should be thoroughly disinfected, preferably by burning.

Tenia Solium.—Tenia solium, the pork tapeworm, resembles Tenia saginata in many respects. The fully grown parasite is 2 to 3 m. long. The *ova* are round or oval, 0.031 to 0.036 mm. in diameter (Braun), with brown shell, radially striated. The oncosphere is about 0.020 mm. in diameter, and possesses six hooklets. The egg is indistinguishable from that of Tenia saginata (Fig. 28, 2) microscopically. The mature *segments* (Fig. 28, 3), which are often seen in the feces, are 10 to 12 mm. long and 5 to 6 mm. broad (Braun). They differ from the segments of Tenia saginata in that the uterus has only 7 to 10 lateral branches extending to either side, and they do not tend to rebranch. The rostellum of Tenia solium is characterized by a double crown of 22 to 32 hooklets, large and small alternating. The head of Tenia saginata, on the other hand, is unarmed.

FIG. 29. — OVUM OF DIBOTHRIOCEPHALUS LATUS. × 460.

Dibothriocephalus Latus.—Dibothriocephalus latus, the fish tapeworm, is the third large cestode frequently parasitic in man. The mature parasite may measure 9 m. in length. The *ova* (Fig. 29) have a rather thin, clear, white or brownish shell, with a small opertulum or cap. The latter stands out particularly well after treatment with

glycerin or dilute sulphuric acid (Blanchard). The eggs are elliptical, and present granular contents. They measure 0.068 to 0.070 mm. by 0.044 to 0.045 mm. (Blanchard). The posterior segments or proglottides (Fig. 30) may be found in the feces, and at times are devoid of ova. Unlike the two preceding parasites, the majority of the segments are broader than they are long, though the reverse may be observed in the posterior segments. The dark brown, rosette-shaped uterus, placed near the center of the proglottis, distinguishes the parasite. The head, which is almond-shaped, is 2 to 3 mm. long and is unarmed. Hooklets are lacking also in the ova.

FIG. 30.—GRAVID PROGLOTTIS OF DIBOTHRIOCEPHALUS LATUS. ×4.

Hymenolepis Nana.[1]—Hymenolepis nana, the dwarf tapeworm, is a very common parasite, especially in children. Because of its small size, infection with this parasite is seldom diagnosed by the finding of segments in the feces. The fully developed parásite is 10 to 45 mm. long and from 0.5 to 0.9 mm. thick. The head is round, 0.25 to 0.30 mm. in thickness, and presents a simple crown of 24 to 30 hooklets. The *ova* are spherical or oval (Fig. 31). The shell is clear and transparent, at times having a light brownish or yellowish tint. It consists of two distinct membranes

FIG. 31.—OVUM OF HYMENOLEPIS NANA. ×460.

[1] Ransom, B. H. "An account of the tapeworms of the genus Hymenolepis parasitic in man, etc." *Bull. no. 18,* Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1904. (A full description of the parasites, with the clinical aspects of infection.)

separated by an intervening space, which contains a transparent substance, more or less finely granular. At two opposite points, usually corresponding to the poles of the egg, there is a small, mammillated projection, often not apparent. To each of these is attached a number of clear hyalin fibers, which pass out through the intermediate substance toward the outer membrane. It frequently happens that the intermediate substance shrinks or retracts from the outer or inner membrane or from both, resulting in the appearance of a third membrane between the two; in reality none exists. The outer membrane is very thin, less than 0.001 mm. The inner membrane is of about the same thickness, and closely invests the oncosphere, which presents three pairs of hooks, usually directed toward one pole (Ransom). The outer dimension of the egg varies between 0.036 and 0.056 mm. long and 0.032-0.042 mm. broad.

FIG. 32.—OVUM OF HYMENOLEPIS DIMINUTA. ×460.

Hymenolepis Diminuta.—Hymenolepis diminuta, commonly found in rats, is occasionally parasitic in man. The parasite is small, being 1 to 6 cm. long and 2.5 to 4 mm. wide. The head, which is unarmed, is 0.2 to 0.6 mm. wide. The *ova* (Fig. 32) resemble those of Hymenolepis nana. They are round or nearly so, and have

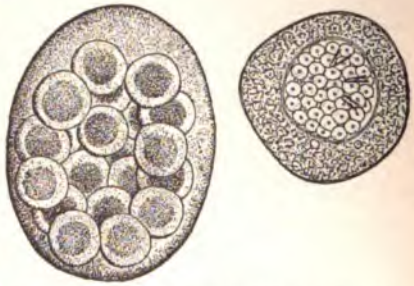


FIG. 33.—DIPYLIDIUM CANINUM, SHOWING AN EGG CAPSULE AND A FREE OVUM. (After Stiles.)

two membranes. The outer membrane may be radially striated. The intervening space between the two membranes is granular. The diameter of the eggs varies between 0.054 and 0.086 mm. (Ransom).

Dipylidium Caninum.—Dipylidium caninum is a common parasite in the intestines of dogs and cats; a number of instances of human infection are recorded.[1] The fully developed parasite is 15 to 35 cm. long. The head is small and the rostellum club-shaped, with three to four rows of hooks, about 60 in number. The mature, gravid segments may be seen without difficulty with the unaided eye. They are 8 to 11 mm. long and 1.5 to 3 mm. broad; their color is often reddish. The genital pores are double, and are opposite. The *ova* (Fig. 33) are spherical, and have a diameter of 0.043 to 0.050 mm. The shell is thin. The uterus contains the eggs in capsules, 8 to 20 eggs being contained in each; they may be encountered in the feces in this form (Stiles). Three pairs of hooks are to be seen in the oncosphere.

Preservation of Gross Specimens of Cestodes and Other Parasites

Stools containing *parasitic ova or embryos* are best preserved by adding commercial formalin (40 per cent.) to a thin watery suspension of the material, so that the latter contains about 2 per cent. of formalin. The majority of eggs are quite well preserved, though thin-shelled ova, such as those of Hymenolepis nana, show considerable distortion. Specimens of feces containing the rhabditiform embryos of Strongyloides stercoralis may be kept for several years; the structure may be wanting in some of the

[1] Lins, J. "Sechs Fälle von Tænia cucumerina beim Menschen." *Wiener klin. Wchnschr.*, 1911, XXIV, 1595.

embryos, but may remain characteristic in others. It is of interest that the formalin does not arrest the development of the ova of Ascaris lumbricoides.[1] The writer has one specimen of feces now more than four years old, preserved with formalin; many of the Ascaris ova contain living embryos. The adult parasites may also be preserved in formalin (2 per cent. solution).

Permanent Preparations of Flatworms

For purposes of study cestode and trematode material may be prepared in several ways.

(1) **Method of Mink and Ebeling.**[2]—The fecal material is mixed with physiological salt solution heated to about the body temperature (37° to 40° C.). The worms move about, and the smaller, such as Hymenolepis nana, are the more readily seen. With forceps the parasites are transferred to a second dish of clear salt solution, in which they become free of mucus and feces. They are now transferred to one of three solutions for fixation: (1) Alcohol, 50 to 70 per cent., with or without glycerin; (2) Zenker's solution (which consists of bichlorid of mercury, 5.0 gm.; potassium bichromate, 2.5 gm.; sodium sulphate, 1.0 gm.; distilled water to 1,000 c. c.), or (3) a 2 per cent. formalin solution; in any of these the material remains 14 to 16 hours. Zenker's fluid causes considerable shrinking and a yellowish discoloration. Formalin is best, since the natural color

[1] Morris, R. S. "The viability of parasitic ova in two per cent. formalin, with special reference to Ascaris lumbricoides." *Johns Hopkins Hosp. Bull.*, 1911, XXII, 299.

[2] Mink, O. J., and Ebeling, A. H. "A method for the preparation of flatworms for study." *U. S. Naval Med. Bull.*, 1909, III, 267.

of the parasite is preserved fairly well with little or no shrinkage. The fixative should be allowed to act not more than fifteen hours, when the parasites are transferred to the following medium:

Syrup (glucose, 48 parts, water, 52 parts)	1,000.0 c. c.
Methyl alcohol	200.0 c. c.
Glycerin	100.0 c. c.
Camphor, q. s. (to keep sterile).	

The specimens may be left in this solution indefinitely, though they are usually sufficiently cleared in 4 to 5 hours. The material is now placed on a slide in glycerin jelly. After the latter has hardened (24 hours or more), the cover glass is sealed with cement.

(2) **Boggs' Method.**[1]—The worm is washed free of feces, and is placed in water or salt solution, in which it is allowed to die, so that it may be fixed while relaxed. It is then placed in a solution of 20 per cent. glycerin in 80 per cent. alcohol, which both fixes and clears the specimen. It is allowed to remain in this fluid in a partially covered dish until the alcohol is entirely evaporated. The specimen is then clear. It is transferred to a glass slide, and the excess of glycerin is removed by blotting paper. Glycerin jelly is then placed on the specimen, which is covered with a cover glass. In 24 to 48 hours, after the jelly has solidified, the preparation is sealed with microscopical cement. To prevent curling of the specimen, it may be spread on a piece of heavy filter paper before immersing it in the glycerin-alcohol solution; it may be necessary to put a light weight on the cover slip until the jelly hardens.

[1] Boggs, T. R. Personal communication, and in Emerson, C. P. "Clinical Diagnosis." 3rd Ed. Philadelphia and London, 1911, p. 444.

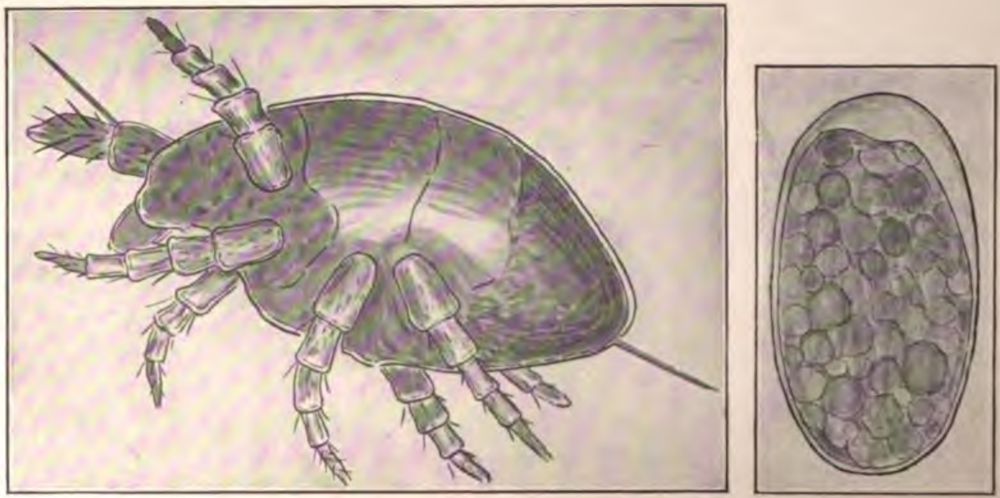


FIG. 34.—TYROGLYPHUS SIRO, the CHEESE-MITE and OVUM. ×460.

Glycerin jelly is prepared as follows:

Gelatin (gold mark).............	14.0 gm.
Distilled water (boiling).........	120.0 c. c.
Dissolve in the hot water and add—	
Glycerin	120.0 c. c.

Cool to 50° C. and add the whites of two eggs. Heat gently without stirring. Strain the mixture through a fine-meshed wire sieve, and filter through cotton while still warm. Add water to make the volume 240 c. c. and 1 c. c. of pure carbolic acid as a preservative. The jelly solidifies on cooling. For use, melt it by immersing the flask containing it in hot water.

(3) **Creosote Method.**—The material is placed in 70 per cent. alcohol, and then in 95 per cent. alcohol, for 15 to 30 minutes. It is then transferred to Beechwood creosote, in which it remains until the tissue is cleared. The time required varies with the size of the specimen and with the degree to which water was withdrawn by the alcohol. Finally, the specimen is mounted in balsam.

Accidental contaminations through food or drink may account for some of the *ova* found in the feces. As an example, the ova of the Tyroglyphus siro (Fig. 34) may be cited. This is the common cheese-mite, which may also be found in flour and other articles of diet. The mites may be found in the stool in addition to the ova. Measurements of the ova may serve to differentiate them from those of intestinal parasites. In cases of doubt, it is advisable for physicians to submit the material to a zoölogist for determination. Such examinations are made at the Hygienic Laboratory, U. S. Public Health Service, Washington, D. C.

CHAPTER IV

THE SPUTUM

In the strict sense of the word, sputum refers to the expectorated material which arises in the respiratory passages between the lung alveoli and the larynx.

To obtain sputum for examination, the patient should be told to discard the nasal and pharyngeal discharges. He should be instructed as to the proper receptacle. In case the physician does not supply a sputum box or cup, the patient may conveniently use a *wide-mouthed* bottle, which has been thoroughly cleansed and sterilized by boiling. In cases where a sputum examination is urgently indicated but no sputum is expectorated, expectorants, such as ammonium chlorid, may be given. Hausmann[1] advises that the fasting stomach be washed out in the early morning with a view to obtaining bronchial mucus; he reports valuable findings with this method. With children it is not infrequently necessary to wash out the stomach, examine the feces, or place the finger, covered with gauze, in the child's throat after a coughing spell and mop out the sputum.

The importance of repeated examinations of the sputa cannot be overemphasized. Particularly when looking for the tubercle bacillus, if the physical signs or history are even suggestive, examinations should be continued. A single negative result means nothing.

[1] Hausmann, T. "Die Frühdiagnose der Lungentuberkulose durch die Mageninhaltsuntersuchung." *Deutsch. Arch. f. klin. Med.*, 1908, XCIV, 595.

Amount.—The quantity of the sputum expectorated in twenty-four hours varies greatly in disease. An approximate estimate of the amount is usually sufficient.

Reaction.—The reaction of fresh sputum is usually alkaline. An old specimen or sputum which has stagnated in the body may be acid in reaction.

Character.—Sputum is designated *mucoid, mucopurulent, purulent, serous,* or *bloody,* as the case may be. The terms are self-explanatory. Various combinations are met with. Bloody sputa may assume any of the shades seen in a bruise. In the presence of jaundice, it must be remembered that green sputa do not necessarily indicate a previous pulmonary hemorrhage; the color is in most cases simply a manifestation of the icterus. Bacterial growth may at times account for a green color.

Odor.—The odor of sputa is important chiefly in connection with putrid affections of the bronchi or lungs.

Consistence.—The consistence of the sputum is generally dependent on the quantity expectorated. With large quantities, the consistence is usually thin, though the sputa of croupous pneumonia form a notable exception. Similarly, when the sputum is small in amount, it is usually more or less tenacious.

Air Bubbles.—Most sputa contain air bubbles. The size of the bubbles is said to indicate roughly the caliber of the bronchi from which the expectorated material is derived. More air is contained in sputum from the smaller bronchi than from the large.

Dittrich's Plugs.—Dittrich's plugs are sausage-shaped casts of the bronchi, varying in size up to that of a white bean. Microscopically, they may be seen to contain fat droplets, fatty acid crystals, cell detritus, and bacteria. A few pus cells and occasionally red blood corpuscles or

hematoidin in granules or needles may be observed, less commonly flagellates.

Bronchial Casts.—Bronchial casts are observed in some diseases with considerable frequency. Their size is determined by that of the bronchi giving rise to them. Casts are usually branched, and consist mainly of fibrin, in which leukocytes, red blood cells, epithelial cells, etc., may be embedded. If the casts are small, their isolation is facilitated by placing the sputum on a white plate, half of which has been painted black. By teasing the specimen with needles, the casts may be found, provided they are macroscopic in size. The addition of water often renders the teasing easier.

Curschmann's Spirals.—Curschmann's spirals occur in various sizes. Only the larger specimens are visible to the unaided eye. The largest spirals measure about 1 mm. in thickness; and are ten to twenty-five times as long. They can only be distinguished as spirals by microscopic examination.

Layer Formation.—When abundant, as in the case of sputa from bronchiectatic cavities, for example, distinct layer formation may be observed. Solid particles collect at the bottom, then a fluid portion, with frothy mucus on the surface. Frequently strands of mucus dip down from the upper layer.

MICROSCOPIC EXAMINATION

Examination of the Fresh Sputum.—Examination of the fresh sputum is greatly neglected. It is as important in the routine study of sputa as the various staining methods, and may furnish information which can be gained in no other way.

For the examination of the fresh specimen two *glass plates* are required, one about the size of the stage of the microscope or a little smaller, the other of the same length or even a bit longer, but about one inch narrower. Old photographic plates, when cleansed, answer the purpose very well; they may be cut into any size desired. In place of the second plate, a glass slide (3x1 in.) may be used. For handling the sputum steel hat-pins are useful. They are inexpensive, and are easily sterilized by heating in the flame of the Bunsen burner.

Some of the sputum to be examined is transferred to the larger plate by means of the hat-pins. It should be so placed on the plate that, when spread out, all parts of the specimen will be accessible for microscopic examination. After sterilizing the hat-pins[1] the second plate or the glass slide is placed over the sputum, which is spread out in a thin layer. Examination is made with the low power objective, for the thickness of the upper plate is usually greater than the working distance of the higher power dry objective. Furthermore, higher magnification is often unnecessary. The magnification may be increased, however, by selecting a strong ocular or by drawing out the tube of the microscope.

After spreading the sputum between the two plates, it is examined macroscopically on a dark background, and any small, opaque masses are noted and singled out for microscopic inspection. Examination then determines whether the particle should be transferred to a slide and studied under a cover glass with higher magnification, or used for staining, or for both purposes. Curschmann's spirals, necrotic tissue fragments, etc., are looked for in

[1] All sputa should be considered as infectious material, and handled accordingly.

this way. The macroscopic examination should always be made, for a great deal of time may be wasted, to say nothing of overlooking important findings, if the microscopic examination is performed aimlessly. There are, of course, specimens in which macroscopic examination reveals nothing, where microscopic study of the preparation is fruitful. But in the majority of instances the naked eye inspection of the thinly spread specimen is an important adjuvant to microscopic examination.

Yellow Elastic Tissue.—Elastic tissue fibers are most easily found by means of the glass plate method. If present, they are often found in yellowish, opaque masses of necrotic tissue about the size of a pinhead or thereabouts. The fibers may present roughly the outline of one or more alveoli (alveolar elastic tissue), single fibers or several long fibers forming a network may be seen (bronchial), or there may be sheets of elastic tissue (arterial). Often only isolated fibers are met with. The yellow elastic fibers are readily seen, and are characterized by (1) their uniform diameter, (2) sharp outline, (3) great refractivity, (4) curling ends, (5) a tendency to branch, and (6) by the fact that pressure on the slide produces no varicosities or thickenings in the fibers.

Fatty acid crystals, which may be mistaken for elastic tissue, usually present varicosities after pressure, and are, furthermore, unlike elastic tissue in that they are soluble in ether or KOH. Again, warming the preparation transforms fatty acid needles into droplets. In contrast to the wavy elastic tissue fibers, fatty acid crystals usually present a single curve.

Curschmann's Spirals.—Curschmann's spirals, often visible macroscopically but never recognizable as such, stand out with distinctness on microscopic examination.

They occur in two forms; in the one there is seen a twisted spiral consisting of delicate, thread-like filaments of mucus, in the turns of which eosinophilic leukocytes, pus cells, epithelial cells, Charcot-Leyden crystals, etc., are caught; in the other form there is a highly refractive central filament, about which the mantle of mucus containing eosinophiles, etc., is twisted. The spirals are subject to much variation in size. Isolated central filaments may be found. The thickness of the filaments differs in different spirals, but in a given specimen it is quite uniform. The finest filaments are extremely minute, while the largest may be twice as thick as a red blood corpuscle.

Fairly satisfactory permanent preparations of spirals may be had by mounting them in glycerin jelly and sealing the specimen with cement after the jelly has hardened.

Dust cells, "heart failure" cells, Charcot-Leyden crystals, and other objects may be seen on examining the sputum on the glass plate. The Charcot-Leyden crystals may be so small as to escape detection. It may, therefore, be advantageous to use a *higher magnification* in their study. For this a selected particle of sputum is transferred to a glass slide and pressed out under a cover glass.

Alveolar epithelial cells, derived from the lung alveoli, are constantly present in the sputum. Their shape varies greatly, since they are possessed of ameboid motion—a fact which is readily demonstrable by examining a perfectly fresh specimen on a warm stage. They are relatively large cells, but are not of uniform size. The nucleus is large and oval. The protoplasm of the alveolar cells is rather coarsely granular, but soon undergoes degeneration, as a result of which fat droplets and myelin granules make their appearance in it.

The *fat droplets* in alveolar cells are, like similar drop-

lets elsewhere, of all sizes, usually round, refractive, and slightly greenish, especially the larger drops. Their nature is determined by adding a drop of alcoholic solution of Sudan III or Scharlach R to the specimen, by means of which fat is stained orange or orange-red.

Myelin degeneration of the alveolar cells gives rise to the macroscopic masses in sputa resembling boiled sago. The myelin droplets may be large or small, and, unlike fat droplets, they are often quite irregular in contour. At times the center of a mass of myelin appears to be thinned. Myelin granules are refractive and have a greenish tint, which is more pronounced than that seen in fat droplets. Myelin is frequently found free in the sputum, probably the result of disintegration or mechanical rupture of the degenerated epithelial cells. It does not take the fat stains.

Dust cells are found in the sputum of all who inhale coal dust. They are alveolar epithelial cells, which have phagocyted the minute particles of coal dust, which constantly are inspired in a smoky atmosphere. The dust appears as dark, brownish-black spots in the cell, which may be so heavily laden that nucleus and protoplasm are entirely obscured. Dust cells are easily distinguished in examining sputum by the plate method. When they are numerous, the sputum is stained more or less diffusely black.

"**Heart-failure cells**" are alveolar epithelial cells which have taken up blood pigment. The name is a misnomer, for they appear in the sputum after a pulmonary or bronchial hemorrhage from any cause whatever. The pigment, which is hematoidin, appears as light, golden-yellow granules, which can scarcely be confused with coal dust.

Red blood corpuscles are often seen in a state of preser-

vation. If the hemorrhage is an old one, however, the cells disintegrate, and only hematoidin in amorphous masses—usually in heart-failure cells—or in needles will be discovered.

Pus cells, polynuclear neutrophilic leukocytes, are always present in the sputa microscopically. The polymorphous nucleus in a cell about 12 micra in diameter with finely granular cytoplasm is characteristic. Occasionally fat droplets are contained in the cells, or they may take up foreign particles in the air passages. In a fresh specimen active ameboid movements may be observed in these cells; pseudopodia are protruded, and the granules of the protoplasm are actively motile.

Eosinophilic leukocytes, of the same size as pus cells and having a polymorphous nucleus, are distinguished by their protoplasmic granules. The latter are coarser than the neutrophilic granules, and are highly refractive, glistening bodies. Ameboid motion may also be observed in these cells. Free granules are usually present in the specimen.

Lymphocytes, or cells which are identical morphologically, are present at times in large numbers. The round or oval nucleus with narrow rim of protoplasm and the small size of the cells—7 to 12 micra—together with the non-granular cytoplasm are distinctive.

Charcot-Leyden crystals are usually found in the sputum with large numbers of eosinophile cells. They are formed wherever eosinophile cells disintegrate. In form the crystals are long lozenges. Their edges are clean cut, the points sharp, and the crystals have a yellowish or greenish tint. They are quite fragile, and the larger crystals may be broken in preparing the specimen. They occur singly or in clusters, and vary greatly in size; the

smallest are visible only with the oil-immersion objective, while large crystals are seen without difficulty with low magnification. On cross-section the crystals are hexagonal. Like the eosinophilic granules, they may be stained with eosin. They are soluble in mineral acids, alkalies, and boiling water. The crystals may be apparently lacking in sputa which contain enormous numbers of eosinophiles. This is particularly apt to be the case when a sputum is first flooded with these cells. If examinations are made from day to day the crystals are found sooner or later.

Crystals of *fatty acid, cholesterin, hematoidin, triple phosphate,* etc., may be encountered in sputa, especially after stagnation. (For morphology and microchemical reactions see the chapter on the urine or feces.)

Microorganisms in Sputa

Only the more important microorganisms of the sputum are referred to in the following pages, and in no case are cultural methods described. For this and details of morphology the reader is referred to works on bacteriology.

Bacillus Tuberculosis

Bacillus tuberculosis may be found in any kind of sputum, for the gross appearance of the sputum in pulmonary tuberculosis is in no way distinctive; it may, in fact, be anything. If the plate examination shows necrotic tissue (elastic tissue), it should be selected for staining, as the bacilli are generally more numerous in such material. Otherwise purulent particles are most suitable for examination.

The preparation for examination is made by smearing the selected particle on a glass slide with a hatpin or other

suitable object. Or it may be pressed between two slides, which are then drawn apart, so that the material is smeared in a thin layer on each of them. In the second way preparations of more uniform thickness are obtained, but there is usually some of the material at the edge of the slide, with which the fingers or other objects may become contaminated. (This is referred to not because it is a valid objection to the method, but because the writer has so frequently observed carelessness in this particular point. Still, one who neglects such an obvious source of infection is sure to make other more serious breaks in technique, and has no business examining infectious material, both for his own safety and, more particularly, for the safety of others.) If the sputum dries slowly, it may be hastened by warming the slide gently. The smear is then fixed in the usual way by passing it through the flame of the Bunsen burner several times.

Ziehl-Neelsen Method.—The Ziehl-Neelsen method of staining is the one generally employed for the tubercle bacillus. The reagents required are:

(1) Carbol-fuchsin.

(a) Fuchsin	1.0	gm.
Absolute alcohol	10.0	c. c.
Dissolve and add—		
(b) 5 per cent. carbolic acid	100.0	c. c.

(2) Acid alcohol.

Hydrochloric acid, conc.	3.0	c. c.
70 per cent. alcohol to	100.0	c. c.

(3) Löffler's methylene blue.

Methylene blue, saturated alcoholic sol.	30.0	c. c.
0.01 per cent. potassium hydrate	100.0	c. c.

Method.—(1) Cover the specimen with carbol-fuchsin and warm it till the stain steams. Maintain this temperature for five minutes.[1] Or the specimen may be immersed in the cold stain for twenty-four hours. It is important to overstain the preparation with carbol-fuchsin, for at best many of the bacilli are decolorized. With light staining, when only a few bacilli are present, they may be missed entirely (L. Brown).

(2) Remove the excess of stain by washing in running water.

(3) Decolorize in acid alcohol, until only the thickest parts of the smear have a faint pinkish tint.

(4) Again wash in water. (Return to the acid alcohol if the specimen becomes pink after washing.)

(5) Stain with Löffler's methylene blue 5 to 20 seconds.

(6) Wash in water, dry the preparation in the air or between sheets of blotting paper. Examine in immersion oil.

The tubercle bacilli are stained red; all else is blue.

Antiformin Method for the Detection of Tubercle Bacilli.—In 1908 Uhlenhuth and Xylander[2] made the important discovery that antiformin possesses the peculiar property of dissolving all bacteria *except* those which are acid-fast, to which class the tubercle bacillus belongs. Applied to the sputum, they found that, in addition to the majority of bacteria, the great mass of the sputum is also liquefied. By the use of antiformin it is, therefore, possible to examine a large quantity of sputum and thus to concentrate the tubercle bacilli present in it. The method is val-

[1] The copper bar used in blood work is convenient for heating the specimen. The stain must be replenished from time to time as it evaporates, to prevent burning the specimen.

[2] Uhlenhuth and Xylander. "Antiformin, ein bakterienauflösendes Desinfektionsmittel." *Berlin. klin. Wchnschr.*, 1908, XLV, 1346.

uable in those cases where the ordinary technique fails to demonstrate bacilli. A number of methods have been described for the use of antiformin, of which the following have been found serviceable:

(1) Löffler's Method.[1]—The quickest method for the use of antiformin with sputum, and one which is well adapted to clinical work, has been described by Löffler. With this procedure the examination may be completed in a comparatively short time. A quantity of sputum (5 to 20 c. c.) is measured, placed in a beaker or flask of Jena glass with an equal volume of 50 per cent. antiformin, and boiled. Solution of the sputum occurs almost at once, the fluid foaming and turning light brown. To 10 c. c. of the cooled solution, which is sterile, add 1.5 c. c. of a mixture composed of 1 volume of chloroform and 9 volumes of alcohol. After shaking thoroughly, the specimen is centrifugalized for about fifteen minutes (the time varies with the speed of the centrifuge). The chloroform is thrown to the bottom of the tube, and on its surface the sediment collects. The supernatant fluid is poured off and the sediment transferred with a clean pipette to a glass slide. The excess of fluid is removed with filter paper, and a small drop of egg albumin (which may be preserved by the addition of 0.5 per cent. carbolic acid) is mixed with the sediment, which is spread on the slide, fixed, and stained in the usual manner. The original sputum may be substituted for egg albumin as the fixative; it is, indeed, preferable, since a more complete examination of the sputum is possible.

The tubercle bacilli are said to be killed with this method (Löffler).

[1] Löffler, F. "Ein neues Anreicherungsverfahren zum färberischen Nachweise spärlicher Tuberkelbazillen." *Deutsche med. Wchnschr.*, 1910, XXXVI, 1987. *Also* Williamson, C. S. "The value of the Loeffler method of sputum examination." *Jour. A. M. A.*, 1912, LVIII, 1005.

As a counterstain Löffler uses malachite green (0.1 per cent. aqueous solution).

(2) PATERSON'S [1] METHOD.—Paterson adds to 10 c.c. of sputum 2.5 c.c. of antiformin,[2] giving a 20 per cent. strength of the latter. If the sputum is very thick or tenacious, or insufficient in quantity, a smaller amount is diluted to 10 c.c. with distilled water.[3] Solution of the sputum occurs rapidly. The mixture is poured into centrifuge tubes, which have been kept in potassium bichromate and sulphuric acid,[4] and rinsed with distilled water just before using. The tubes are stoppered with unused corks, shaken vigorously, and set aside for 24 hours at room temperature or for 4 to 6 hours at 37° C. The tubes are again shaken and then centrifugalized. The supernatant fluid is poured off, the tubes refilled with sterile physiological salt solution, again corked, shaken, and centrifugalized. The washing is done a second time to rid the sediment of all alkali; otherwise it does not adhere well to the glass. The sediment is then transferred to a slide, smeared, fixed, and stained by the Ziehl-Neelsen method.

[1] Paterson, R. C. "A report on the use of 'antiformin' for the detection of tubercle bacilli in the sputum, etc." *Jour. Med. Research*, 1910, XXII, 315.

[2] The composition of antiformin, according to Paterson, is equal parts of 15 per cent. solution of sodium hydrate and of liquor sodæ chlorinatæ (B. P.). The latter is prepared as follows:

Sodium carbonate	600.0 gm.
Chlorinated lime	400.0 gm.
Distilled water	4,000.0 c. c.

Dissolve the sodium carbonate in 1.000 c.c. of distilled water. Triturate thoroughly the chlorinated lime in the remainder of the water. Filter. Mix the two and filter again. There is formed an alkaline, almost colorless liquid with a strong odor of chlorin. It keeps well.

[3] Before this is done, the existence of acid-fast bacilli in the distilled water should be excluded—a troublesome source of error at times, as shown by W. Brem (*Jour. A. M. A.*, 1909, LIII, 909).

[4] Rub up some potassium bichromate with sulphuric acid for two minutes, allowing the acid to take up as much of the bichromate as it will. Pour off the acid and repeat the process.

The washing with salt solution may be dispensed with. The sediment, which consists of débris, swollen and distorted cells, etc., is fixed to the slide with difficulty because of the alkali, but this may be overcome by first smearing the slide with some of the original sputum or with egg white (preserved by the addition of 0.5 per cent. pure carbolic acid). The original sputum is to be preferred, since the other elements present in it may thus be studied.

The *tubercle bacilli are not killed* and the sediment should, therefore, be handled with the usual precautions.

The method is valuable for obtaining material for animal inoculation or for cultures.

(3) BOARDMAN'S METHOD.[1]—The following procedure has been found satisfactory by Boardman: Fifteen to 20 c. c. of sputum are placed in a conical specimen glass, and antiformin is added sufficient to make a 20 per cent. strength of the latter. After the solution has become homogeneous and watery in consistence, an equal volume of 95 per cent. alcohol is added. By this means sedimentation is facilitated, since the specific gravity of the mixture is less than 1.000. After stirring, allow it to stand till sedimentation is complete. The clear supernatant fluid is poured off (into a disinfecting solution), and smears are made of the sediment on a glass slide, using some of the original sputum as a fixative. The specimen is then fixed with heat and stained in the usual way.

Diplococcus Pneumoniæ

The *pneumococcus,* found not infrequently in the upper respiratory passages of healthy individuals, is often con-

[1] Boardman, W. W. "The use of antiformin in the examination of the sputum for the tubercle bacillus." *Johns Hopkins Hosp. Bull.,* 1911, XXII, 269.

spicuous in the sputum of acute lobar pneumonia and other conditions. It is a Gram-positive organism, whose capsule may be seen after staining with Gram's method.

Gram's Method of Staining.

Reagents: 1. Anilin water gentian violet.

Ten c. c. of anilin oil are shaken with 100 c. c. of distilled water till a milky emulsion is secured. After standing five minutes, it is filtered through a *wet* filter. To the filtrate, which should contain no large oil drops, add 11 c. c. of saturated alcoholic solution of gentian violet and 10 c. c. of absolute alcohol. The solution keeps not more than 8 to 10 days (Schmorl).

2. Gram's iodin solution:

Iodin	1.0 gm.
Potassium iodid	2.0 gm.
Distilled water	300.0 c. c.

Method.—(1) Stain the heat-fixed smear in anilin water gentian violet 1 to 3 minutes.

(2) Wash quickly in water.

(3) Cover the specimen with Gram's iodin solution about $1\frac{1}{2}$ minutes.

(4) Decolorize in absolute alcohol until the preparation has a grayish or yellowish color—usually about 5 minutes. (The specimen may now be dried and examined. If a counterstain is desired, the further steps are carried out.)

(5) Wash in water.

(6) Stain with 0.2 per cent. aqueous solution of Bismarck brown 1 minute.

(7) Wash in water, dry in the air or blot, and examine in immersion oil.

The pneumococci and all other Gram-positive organisms

are stained blue, the remaining bacteria and cell nuclei are brown.

The pneumococcus or Diplococcus pneumoniæ grows in pairs. The long axes of the organism are placed end to end.

For demonstration of the capsules, Welch's method may be employed.

Welch's Capsule Stain.—(1) Flood the fixed smear with glacial acetic acid, and immediately pour it off.

(2) Wash off the acid with anilin water gentian violet.

(3) Wash in 2 per cent. sodium chlorid solution, and examine the wet specimen.

Bacillus Influenzæ

Bacillus influenzæ is a very minute bacillus, which often exhibits polar staining. It may be found free in the sputum or within pus or epithelial cells. It decolorizes by Gram's method of staining and, therefore, takes the counterstain. It may be *stained* satisfactorily with carbol-fuchsin. The stain is diluted 1:10 with distilled water, and allowed to act for ten minutes or longer. Cultural methods are required for the complete identification of Bacillus influenzæ. It grows on media containing blood.

Bacillus Diphtheriæ

Bacillus diphtheriæ, though not usually found in the sputum, may be considered here. In examining for it the membrane is brushed with a sterile swab, with which, after inoculating tubes of Löffler's serum, smears may be made on glass slides. The smears may show the organism, but in any case it is better to examine a culture on blood serum which has been incubated at body temperature for 9 to 18

hours. From a fresh culture, of the age given above, the organisms exhibit polar staining. Usually two deeply staining bodies are seen in a bacillus at either pole, though there may be but one or as many as three. Chains are for the most part lacking; frequently the bacilli are placed with their long axes parallel. The polar staining is beautifully demonstrated with Neisser's method.

Neisser's Staining Method.

Reagents:

(1)	Methylene blue	1.0 gm.
	Alcohol, 90 per cent.............	20.0 c. c.
	Dissolve and then add—	
	Distilled water	950.0 c. c.
	Acetic acid, glacial..............	30.0 c. c.
(2)	Vesuvin (Bismarck brown)......	2.0 gm.
	Boiling distilled water...........	1,000.0 c. c.
	Dissolve. Filter after the solution cools.	

Method.—(1) Stain in methylene blue solution 1 to 3 seconds.

(2) Wash quickly in water.

(3) Stain in vesuvin 3 to 5 seconds.

(4) Wash quickly in water, blot dry, and examine in oil.

The diphtheria bacilli are slender rods, often having a slight bend. They are stained light brown, with one to three granules, which take a dark blue color. Parallel pairs of bacilli are characteristic. Chain formation is lacking. Other bacteria, which are present in the smear, are stained light brown, so that the blue polar bodies of Bacillus diphtheriæ are striking and characteristic.

Beall's Method.[1]—The polar bodies may be demonstrated well by Beall's method.

(1) Overstain the specimen with anilin water gentian violet 1/4 to 1/2 minute.

(2) Wash in water.

(3) Decolorize with 10 per cent. glacial acetic acid till little color remains. This is controlled under the microscope.

(4) Wash in water, blot dry, and examine in immersion oil.

All Gram-negative organisms are decolorized, and most of those which are Gram-positive. The polar staining is intense, and diphtheria bacilli stand out prominently. Practically all other organisms are decolorized more or less completely.

Actinomyces Bovis

Actinomyces bovis (Fig. 35), the ray fungus, which is the causative agent in "lumpy jaw" of cattle, is occasionally parasitic in man. The lungs are often the site of infection.[2] The sputum is characteristic. Claypole studied the series of cases reported by Bridge, and describes the sputum as follows: "In the majority of cases the sputum is characteristic and of two types: (1) glairy, mucilaginous, often quite watery; (2) purulent, more or less bloody, more or less—sometimes intensely—fetid. Both types may be found sparingly or in abundance. . . . The small granules (of the fungus), usually the size of a very small pinhead, can be picked out with a

[1] An unpublished method of Dr. H. K. Beall of Fort Worth, Texas, through whose kindness it is given here.

[2] For a review of the subject, see Bridge, N. "Streptothricosis (actinomycosis) of the lungs." *Jour. A. M. A.*, 1911, LVII, 1501.

needle and put on a slide for examination. They are quite tough, and can be washed free of débris by putting them in a dish of water and squirting them vigorously up and down a pipette. . . . "

"Under low magnification the yellow color is marked; to the naked eye the fungus is grayish-white. The edge is always darker, even shading into a brown; toward the center it grows lighter. From this light, almost homogeneous center, the characteristic radiations arise. Higher magnification shows the center to be a mass of pale, radiating threads, the mycelia, and at the edges a mass of threads and cocci. Both mycelia and cocci may be stained with methylene blue, the former frequently being banded light and dark in segments, sometimes granular throughout."

FIG. 35.—ACTINOMYCES HOMINIS, SHOWING CLUB-SHAPED EXTREMITIES TO THE RAYS. (Fresh preparation.) (After Wood.)

Streptothrix Eppengeri

Streptothrix eppengeri is an organism related to the preceding. In the case described by Warthin and Olney[1] a filamentous, branching organism was found. Usually the mycelia were tangled and interwoven. No conical or club-shaped terminations were found, as in Actinomyces bovis. The threads, stained with carbolfuchsin, were not decolorized after treatment with 25 per cent. nitric or sulphuric acids, though the stain was largely removed by washing in 95 per cent. alcohol.

[1] Warthin, A. S., and Olney, H. S. "Pulmonary streptothricosis." *Amer. Jour. Med. Sci.*, 1904, CXXVIII, 637.

Blastomycetes

Blastomycetes (Fig. 36) have been found in the sputum in a number of cases. "In unstained preparations of pus and tissue the organisms appear as round or oval

FIG. 36.—BLASTOMYCETES IN SPUTUM. × 1500. Photomicrograph. (After E. E. Irons and E. A. Graham.)

bodies with a double contoured, highly refractive capsule. Within the capsule, in many instances, granules or spore-like bodies can be distinguished. The addition of a 1 to 10 per cent. solution of potassium hydrate to the specimen

under examination facilitates the recognition of these bodies. In stained sections the double-contoured, homogeneous capsule is usually separated from a finely or coarsely granular protoplasm by a clear space of varying width. Vacuoles of different sizes are found in some organisms. In both pus and tissue organisms in pairs or in various stages of budding are commonly seen. The parasite, as a rule, varies in size from 7 to 20 micra, though slightly smaller and much larger forms occur in some cases."[1]

Animal Parasites in the Sputum

In this country animal parasites are comparatively rare in the respiratory passages, though probably more common than is generally supposed.

Entameba histolytica (Fig. 12) may be encountered in the sputum as the result of rupture of an amebic liver abscess into the bronchial tree. The organism is identical with that found in the feces (q. v.). Perfectly fresh sputum should be examined—when possible, with a warm stage.

Fig. 37.—Ovum of Paragonimus westermanii from the Sputum. ×400. (After Emerson.)

Entameba tetragena (Fig. 12) occurs in the sputum under the same conditions as Entameba histolytica.

Trichomonads (Fig. 13) and *cercomonads* are occasionally found in the sputa, usually in material which has stagnated in the lung.

Paragonimus westermanii, the lung-fluke, the cause of "parasitic hemoptysis," is rare in this country, common

[1] Montgomery, F. H., and Ormsby, O. S. "Systemic blastomycosis." *Arch. Int. Med.*, 1908, II, 1. See also Hektoen, L. "Systemic blastomycosis and coccidioidal granuloma." *Jour. A. M. A.*, 1907, XLIX, 1071. (Literature.)

in Japan and parts of China. Diagnosis is made by finding the ova (Fig. 37) in the fresh sputum (see p. 194).

Echinococcus cyst, though common in certain parts of the world, is excessively rare in this country. Diagnosis

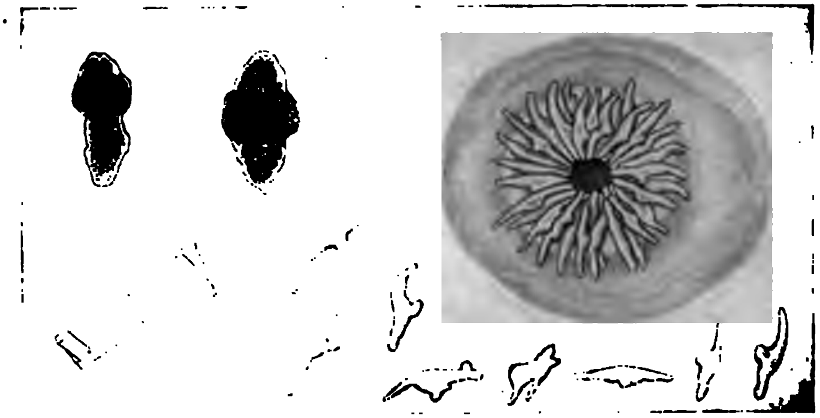


FIG. 38.—SEDIMENT FROM ECHINOCOCCUS CYST. Above and to the left are two degenerated scolices (× about 60); to the right is a crown of hooklets (×400); below are hooklets of unusual shapes and a small mass of cholesterin crystals (×400). (After Emerson.)

may be made after rupture of the cyst into the bronchi by (1) the finding of daughter cysts, (2) scolices, (3) hooklets, or (4) parts of the membrane in the sputum (Fig. 38). The material may arise from a hepatic cyst which has ruptured through the diaphragm into the air passages.

CHAPTER V

THE BLOOD

Obtaining Blood for Examination.—Blood for counts, etc., is obtained most conveniently from the lobe of the ear or the ball of the finger. The ear is, on the whole, more satisfactory than the finger. It is easily accessible, the flow of blood is as good, and it is much less sensitive to pain than the finger. There is also less likelihood of infection of the small wound through contact with dirty objects. For counts or hemoglobin determinations, blood should ***not*** be drawn from a part of the body which is cyanotic, because concentration of the blood may occur, producing results which are misleading (too high).

Blood Stickers.—A number of satisfactory blood stickers are on the market, and require no special description. In default of these a Hagedorn needle may be used. Bass [1] has recently described a simple arrangement consisting of a straight surgical needle mounted in a cork. When not in use, the needle is carried in a small vial filled with alcohol. A sharp steel pen, one of whose prongs has been broken off, may be employed as a sticker. The sticker should always be perfectly clean, in addition to being sterile. Dried blood on the point, even a very small amount of it, makes a sharp instrument seem dull.

Method.—The skin of the ear or finger and the sticker are cleaned with alcohol or ether, which is allowed to evapo-

[1] Bass, C. C. "A practical, inexpensive, aseptic blood-sticker." *Med. Record*, 1910, LXXVIII, 538.

rate completely. The skin is then pierced. The point of the sticker should be held close to the skin and pushed in rather quickly; beginners frequently make a sudden stab at the part from a distance of several inches, either missing the skin entirely or producing an unnecessarily deep wound. The wound must be such that the blood flows freely from it; squeezing the tissues to obtain blood is not permissible, since the blood is thereby diluted with lymph.

COUNTING THE BLOOD CORPUSCLES

The Hemocytometer.—In blood counting the standard instrument in universal use is the hemocytometer of Thoma

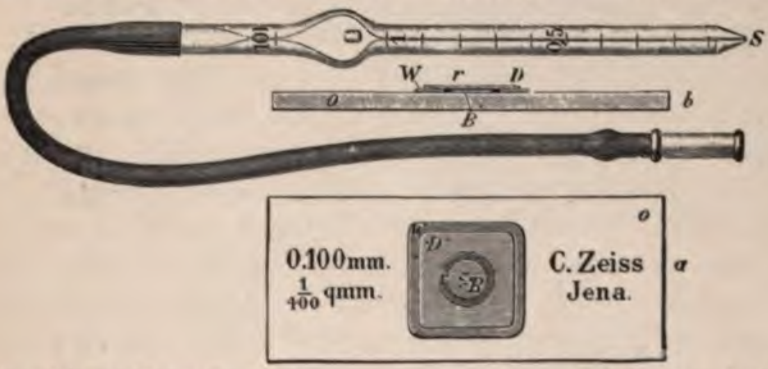


FIG. 39.—THE THOMA-ZEISS HEMOCYTOMETER. *a*, the counting chamber; *b*, the same in section; *S*, the diluting pipette.

(Fig. 39). The instrument as originally designed by Thoma was not satisfactory for the enumeration of the leukocytes, and, as a result of this, numerous modifications of the Thoma ruling of the counting chamber have been brought forward.[1] The change consists in an increase of

[1] The writer learns from dealers in laboratory supplies that they are forced to keep the original counting chamber in stock, since physicians specify "Thoma-Zeiss" in ordering. This is doubtless due to the fact that the purchasers are unfamiliar with the much more practical rulings—modifications of the Thoma—which are mentioned above.

the ruled area from 1 sq. mm. to 9 sq. mm. Neubauer, Türk, Zappert-Ewing, and others have devised rulings, which are designated by their names. The writer prefers the Neubauer ruling (Fig. 40). In all the modifications the central square millimeter retains the original ruling of Thoma. The additional eight square millimeters surround it, and are solely for greater accuracy and convenience in counting the white corpuscles.

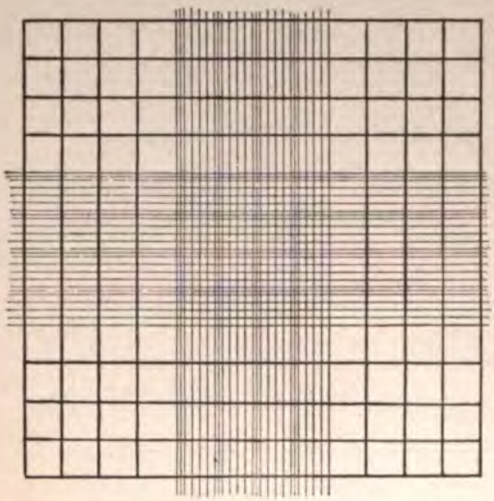


FIG. 40.—THE NEUBAUER RULING OF THE HEMOCYTOMETER.

THE RULING OF THE COUNTING CHAMBER.—There is, then, a ruled area 3 mm. on a side or 9 sq. mm. (Fig. 40). This area is divided into nine large squares, each of which is 1 mm. on a side or 1 sq. mm. The *central* square millimeter, which is used for counting the erythrocytes, is subdivided into 400 small squares, each of which is $\frac{1}{20}$ mm. on a side, and has, therefore, an area of $\frac{1}{400}$ sq. mm.[1] By means of double lines these smallest squares are grouped into blocks of twenty-five, a convenient unit to employ in counting. The ruling of the *remaining eight large squares* (1 sq. mm. each) varies according to the design selected. For the leukocyte count the entire nine square millimeters may be used.

[1] This is always indicated on the glass slide of the counting chamber—"$\frac{1}{400}$ qmm." The depth is also given—"Tiefe 0.100 mm."

Construction of the Counting Chamber (Fig. 39).—The ruling is on a glass disc (B) which is mounted on a heavy glass slide (o). The disc is surrounded by a glass table (W), also attached to the slide, the surface of which is exactly one-tenth (0.1) of a millimeter above that of the ruled disc. A moat (r) about 2 mm. wide separates the disc from the table. When the cover glass (D), which is supplied with the apparatus, is placed upon the glass table, it thus forms a space between its under surface and the surface of the ruled disc, which is 0.1 mm. deep.

The Diluting Pipettes.—Since whole blood is much too thick to permit direct enumeration of its corpuscular elements, pipettes with which accurate dilutions of the blood can be made are required. Two pipettes are furnished with the complete hemocytometer, one for the red cells, the other for the white. Each consists (Fig. 39) of a capillary tube, which opens into a bulb containing a glass pearl. The capillary tube is divided into 10 equal parts. In the red pipette the bulb, when filled to the line on its upper outlet (marked 101), holds one hundred times the contents of the ten divisions of the capillary tube. It is, therefore, possible to obtain ten different dilutions of blood, if desired. Practically, only two dilutions are employed—1:200 and 1:100.

With the *white pipette* lower dilutions are made. The bulb usually contains either ten or twenty times the content of the capillary tube. Dilutions of 1:10, 1:20, etc., are generally made.

Procedure in Counting the Erythrocytes

(1) **Diluting Fluids.**—The requirement for the diluting fluid is that it preserves well the red corpuscles. Nu-

merous formulæ have been elaborated. Among the better known of these are the following:

(a) *Hayem's solution*:

Bichlorid of mercury	0.5 gm.
Sodium chlorid	1.0 gm.
Sodium sulphate	5.0 gm.
Distilled water	200.0 c. c.

Dissolve and preserve in a tightly stoppered bottle.

This is the most satisfactory diluting fluid. It keeps indefinitely, and no organisms grow in it. The red cells settle evenly in it.

(b) *Physiological salt solution*:

Sodium chlorid	0.85 gm.
Distilled water	100.0 c. c.

Dissolve.

The red cells settle slowly and often unevenly in salt solution.

(c) *Toisson's fluid*:

Sodium sulphate	8.0 gm.
Sodium chlorid	1.0 gm.
Glycerin (neutral)	30.0 c. c.
Distilled water	160.0 c. c.
Methyl violet	q. s.

Dissolve. (The methyl violet is added in minute amount (25 to 30 mg.), just enough to color the fluid, which should remain clear and

Toisson's fluid is not stable and must be filtered before using. Low forms of vegetable life luxuriate in it, and it is, on the whole, an unsatisfactory fluid, its only advantage —which is usually negligible—being that leukocytic and other nuclei are stained.

(2) **Filling the Pipette.**—The first essential is to have the blood flowing freely and to obtain a fresh drop. If it is necessary to squeeze the ear to obtain the blood, the latter will be diluted with tissue lymph, making the count too low; if the drop is not perfectly fresh, clotting will have begun, so that a uniform suspension of the cells cannot be secured. The blood is sucked cautiously into the capillary tube to the line marked 0.5. (With anemias of 2,500,000 cells or less, the blood is more conveniently drawn up to the mark 1 to secure a lower dilution, i. e., 1:100.) If the blood is accidentally sucked above the line, it may be lowered by drawing the finger across the tip of the pipette, *provided* the column of blood has not passed more than 1 mm. above the line; if it has extended farther, the blood adhering to the wall of the tube will be sufficient to introduce a serious error in the dilution. In the latter case the blood should be drawn up to the next line on the capillary tube, 0.6, quickly, and a corresponding correction in dilution calculated. Bubbles of air in the column of blood must, of course, be avoided. After the tube has been accurately filled with blood, the end of the pipette is wiped free of blood, and is then plunged into the diluting fluid, which is sucked up to the line marked 101. While the diluting fluid is being drawn up, the pipette, held between thumb and fingers, is revolved to keep the glass pearl within the bulb in motion, both for the purpose of mixing the blood and diluting fluid and also to prevent bubbles adhering to the pearl, which would render the dilution inaccu-

rate. The filling of the capillary tube and the subsequent dilution of the blood require rapid manipulation to prevent clotting. When the fluid reaches the line 101, the mouthpiece of the pipette is occluded with the tongue. The finger is then placed over the tip of the capillary tube, the thumb grasping the other end of the pipette. An even and uniform suspension of the erythrocytes is now secured by shaking the pipette, held *horizontally,* for at least two minutes. After the shaking is completed, several drops are blown out of the pipette to thoroughly empty the capillary tube (which contained only diluting fluid), and the counting chamber is then filled. Since the red corpuscles settle quite rapidly, the contents of the pipette must be perfectly mixed by shaking each time before filling the counting chamber.

(3) **Filling the Counting Chamber.**—The counting chamber and cover glass must be perfectly clean and free from dust. A drop of the diluted blood is placed at one side of the ruled disc; the cover glass, used as a lever, is gradually lowered onto the drop, the edge of the glass table serving as the fulcrum. As the cover glass comes in contact with the drop, and the latter spreads over the surface of the ruled disc, there will be a tendency for one or more bubbles to form. By alternately raising and lowering the cover glass the fluid will spread evenly, and bubbles can be avoided. The size of the drop of diluted blood which is taken is important, and is learned by practice. It should be just large enough to cover the surface of the ruled disc, when the cover glass is applied. If it is so large that much of the fluid runs into the moat or that any fluid is found between the cover glass and glass table, the counting chamber must be cleaned and refilled. Before proceeding to count the erythrocytes, two conditions must be fulfilled

in addition to those already given: (1) Newton's rings (prismatic colors) must be visible between the cover glass and glass table when looked at obliquely toward the light, since this proves that the two surfaces are in close apposition and are not separated by a particle of dust, which would deepen the chamber; and (2) inspection under low magnification should show no clots and no gross inequalities in the distribution of the red cells over the ruled surface. These conditions being fulfilled, after allowing the erythrocytes to settle in the fluid two or three minutes so that all of them will be in the same focus, the enumeration of the cells is proceeded with.

In making the count it is advisable, if the worker is inexperienced, to use a high magnification (Leitz ocular 1, objective 6, or corresponding values for other makes of microscope), which will include a single block of twenty-five small squares. Ordinarily, however, a lower magnification is sufficient, such, for example, as one obtains with Leitz ocular 3, objective 3, with the tube drawn out to its full length.

(4) **The enumeration of the cells** may be made in an almost endless number of ways. The method described by Emerson [1] has been employed by the writer, and is as follows: The cells in 200 small squares are counted. This is accomplished by counting separately eight blocks of twenty-five small squares, the blocks used being those at the corners of the ruled area from each of two preparations. A little more time is consumed in refilling the counting chamber, but it serves as an additional check on the accuracy of one's technique. Beginning at the upper left corner of a block of twenty-five small squares, the count is made from left to right in the upper tier of five small

[1] Emerson, C. P. "Clinical Diagnosis."

squares, then from right to left in the second tier, and so on, until the entire block of twenty-five has been covered. The *cells touching the line* on two adjacent sides of a square are counted, while those on the line of the two remaining sides are disregarded. The total count for each block of twenty-five small squares is set down. When all eight blocks have been counted, the difference between the highest and lowest total count should not exceed 25 cells. If this limit is exceeded, the distribution of the cells in the counting chamber has been so uneven as to make the results untrustworthy.[1]

(5) **Calculation of the Result.**—The calculation is simple. Since each small square is $\frac{1}{20}$ mm. on a side, and the counting chamber is $\frac{1}{10}$ mm. deep, the cubic content of one small square is $\frac{1}{20} \times \frac{1}{20} \times \frac{1}{10}$ or $\frac{1}{4000}$ c. mm. As 200 such squares have been counted, it follows that the sum of all the cells counted is the number of cells contained in $\frac{200}{4000}$ or $\frac{1}{20}$ c. mm. of *diluted* blood. Since the dilution used was 1:200, the total number of cells counted must be multiplied by 20 and by 200, or by 4,000, to obtain the number of cells in 1 c. mm. of *undiluted* blood, the desired result.

The normal count usually given for healthy adults is: for males, 5,000,000; for females, 4,500,000 cells per c. mm. These figures represent averages. The red count of normal adults is not infrequently as high as 6,000,000.

(6) **Cleaning the Apparatus.** (a) *The Counting Chamber.*—The counting chamber should be cleaned with *water* [illegible]. After [illegible] it is wiped dry with a soft, clean cloth. The [illegible] blood should never be permitted to dry [illegible] chamber. [illegible], ether, or similar

[1] [illegible] conditions and [illegible] more than [illegible]

solvent should not be employed, as it may dissolve the cement, by which the ruled disc and the glass table surrounding it are fastened to the slide. (In case this accident happens, all the parts may be returned to the maker, for it is possible at times to repair the damage.)

(b) *The Pipettes.*—The pipettes should be cleaned immediately on completion of the count. If this is impossible, they should at least be emptied and refilled with water, until it is convenient to clean them. If allowed to stand, the diluted blood drying in the tip of the capillary may occlude it. The successive steps are as follows:

(1) The pipette is emptied of its contents.

(2) Distilled water or clear tap water is drawn into the pipette. After emptying, fill it with—

(3) Ethyl alcohol, 95 per cent. Shake the pipette so that the water adhering to the pearl is mixed with the alcohol, place the rubber tubing over the tip of the capillary tube, and blow the alcohol through.

(4) Fill the pipette with ether, and shake again. Remove the rubber tubing, and allow the ether to run out by inverting the pipette.

(5) Aspirate air through the pipette till the walls of the bulb are dry and the glass bead rolls freely as the pipette is rotated. If the bead sticks to the wall, it means that there is still moisture remaining, or that the pipette is not clean.

A suction pump is a great convenience in cleaning the pipettes. As a substitute for the pump, a stiff-walled rubber bulb may be employed.

If blood clots in the capillary tube, it should be removed as soon as possible by means of a horse hair. For this purpose hairs from the tail or mane are washed in water and alcohol and kept in the latter. Wire should never be

used, as it may scratch the glass, and there is also great danger of chipping the end of the pipette. At times, if the clot has become very firm or dry, it is necessary to place the pipette in a test tube with nitric acid. When a film of coagulated albumin forms over the inner surface of the bulb, the pipette may be filled with nitric acid and set aside for several hours.

Counting the Leukocytes

(1) **Diluting Fluid.**—For counting the white cells of the blood it is necessary to have a fluid which will render the erythrocytes invisible and cause the leukocytic nuclei to stand out prominently. Dilute acetic acid is universally employed for this purpose. It is used in about 1 per cent. strength. The solution is quickly prepared by adding two drops of glacial acetic acid to 10 c. c. of distilled water. The dilute acid should be prepared freshly each day; yeast cells grow in it, if it is kept, and may lead to confusion, since single cells resemble the nuclei of lymphocytes, while several cells are not very unlike a polymorphous nucleus. It is convenient to have a small, wide-mouthed bottle with a file-scratch indicating 10 c. c. for preparing the dilute acetic acid.

(2) **Filling the Pipette.**—The capillary tube of the white pipette is larger in caliber than that of the red pipette, and, therefore, a larger drop of blood will be required to fill it. The blood is sucked up to the mark 0.5, as a rule; the blood on the end of the pipette is wiped off, and the tip immediately plunged into the diluting fluid, which is sucked up to the mark 11 (or 21 in the case of the larger white pipettes). The pipette must be rotated more jerkily and the fluid sucked in more slowly than with the red

pipette, to avoid the air bubble which so often clings to the glass pearl. After the pipette is filled the end is occluded, and the pipette, held horizontally, is shaken at least two minutes. In short, all the precautions requisite to a proper filling of the red pipette (to which the reader is referred) apply with equal force here. Bubbles in the column of blood or in the bulb of the pipette ruin the preparation. The column of blood must be drawn quickly and accurately to the desired mark.

When the number of leukocytes is greatly increased, as is the case in extreme leukocytoses and usually in leukemia, it is often more convenient to use the red pipette for the leukocyte count in order to obtain a greater dilution.

(3) **Filling the Counting Chamber.**—The counting chamber is filled in the manner described under the red cell count. Since the capillary tube of the white pipette is wider, the diluted blood flows out of it more rapidly, and the size of the drop is less easily controlled. A drop of the right size, Newton's rings, and an even distribution of the cells over the ruled area are essential.

The magnification employed should be the same as that used in counting the erythrocytes (q. v.). Work is much more rapid with the lower power.

(4) **The Enumeration of the Leukocytes.**—After the cells have settled until all are in the same focus (usually in two or three minutes), the leukocytes, whose nuclei stand out as refractive bodies, are counted in one square millimeter at a time, including the cells which touch the line on two sides of the square only. Nine or ten square millimeters are counted—nine in one preparation, or five in each of two preparations. The procedure is the same as that used in counting the red corpuscles, the only difference, being the larger unit employed, 1 sq. mm. The difference

17

between maximal and minimal total count for 1 sq. mm. should not exceed 8 cells.

The normal leukocyte count of adults lies between 5,000 and 10,000 cells per c. mm.—rarely 12,000 cells.

If the diluting fluid is not freshly prepared, ***yeast cells*** may be counted and lead to serious error. A second source of error is the presence of a considerable number of *nucleated red cells* in the blood. Ordinarily erythroblasts are detected only when the stained blood is simultaneously examined; and, as they are numerous only in marked pathological states of the blood, they seldom escape notice. They cannot well be separated from the leukocytes in the counting chamber, so that the count obtained is the sum total of all nucleated cells in the blood—both red and white. The number of erythroblasts is determined by making a differential count of the stained blood and noting the relative number of nucleated red cells as compared with the leukocytes. From the proportion of the two kinds of cells found, the correction of the white count is made. Thus, if the leukocyte count were 20,000, and differential count showed 125 nucleated reds to 500 leukocytes, the relative frequency of the two would be as 1:4. Therefore, there are present in one cubic millimeter of blood 5,000 nucleated red cells. Since these were included with the leukocytes in the total count, the latter must be corrected by deducting the nucleated reds. The leukocyte count thus becomes 15,000 instead of 20,000.

(5) **Calculation of the Result.**—Let us assume that the dilution employed was 1:20, and that nine square millimeters were counted. Each square millimeter has a cubic content of 0.1 c. mm., since the counting chamber is 0.1 mm. deep. The sum of the leukocytes in the nine large squares divided by 9 gives the average number of cells in 0.1 c. mm.

of *diluted* blood. To obtain the number of cells in 1 c. mm. of *undiluted* blood, this number is multiplied by 10 and by 20 (dilution 1:20), or by 200.

Bürker's Modification [1] of the Thoma Counting Chamber.—By substituting two wedge-shaped, ruled glass plates for the ruled disc, Bürker has modified Thoma's design.

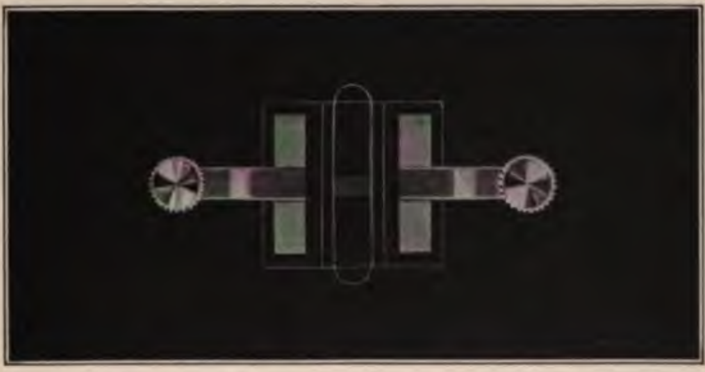


FIG. 41.—BÜRKER'S HEMOCYTOMETER. (Zeiss.)

The wedges are placed with their bases opposite one another (Fig. 41), the apices extending out toward the sides of the glass slide. Two oblong tables at either side of the wedges with long axes running parallel replace the glass table which surrounds the ruled disc in the Thoma pattern. The cover glass, when resting on the tables,[2] is 0.1 mm. above the ruled surface of the wedges, as in the Thoma apparatus. The ruling is after a special design of Bürker.[3] Each wedge is ruled, and the chamber has, there-

[1] Bürker, K. "Erfahrungen mit den neuen Zählkammer nebst einer weiteren Verbesserung derselben." *Arch. f. d. ges. Physiol.*, 1907, CXVIII, 460.

[2] When ordered, clips are supplied with the apparatus to hold the cover glass firmly in place.

[3] By special order Zeiss makes the Bürker counting chamber with Neubauer ruling.

fore, *two ruled areas* in place of the one of the Thoma chamber.

In filling the Bürker hemocytometer for counting, the first step consists in placing the cover glass on the tables, Newton's rings being obtained. Then the blood is allowed to run under the cover from the pipette by capillarity. One side may be filled for the red count, the other for the white.

Bürker calls attention to the following technical points which should be observed: (a) To avoid bubbles, the cover glass and chamber must be carefully cleaned. Furthermore, in using pipettes having an angle at their tips, bubbles are prevented with difficulty; the tip should be rounded off with emery paper. (b) The drop of blood which flows under the cover glass should not be so large as to overflow into the gutter. Counting chambers should not be accepted in which the gutter between the two ruled surfaces is less than 2 mm. wide and the lateral gutters 1.5 mm. wide. (c) After allowing the red cells to settle for at least three minutes, the evenness of distribution should be determined before proceeding with the count. Bürker advises that this be accomplished in the following manner: The counting chamber is placed on the stage of the microscope, illuminated by the mirror with the diaphragm opened wide. By viewing the counting surface obliquely with the unaided eye one sees a film or veil formed by the erythrocytes. Irregularities in the distribution of the cells are shown by variations in the density of the film. When such irregularities are visible, the chamber must be refilled. In counting the leukocytes, this procedure is not applicable, for the cells are too few. Microscopic examination with low power must be made.

Counting the Eosinophilic Leukocytes

The number of eosinophilic cells is usually arrived at by making a total count of the leukocytes in the ordinary way and, at the same time, preparing stained specimens. By finding the percentage of eosinophiles in a differential count, the absolute number of cells per cubic millimeter may then be calculated.

Dunger[1] has devised a method by which the absolute number of eosinophiles per c. mm. may be determined directly.

Dunger's Method.—The formula of the diluting fluid is:

1 per cent. aqueous solution of eosin..	10.0 c. c.
Acetone	10.0 c. c.
Distilled water	90.0 c. c.

The solution must be preserved in a tightly corked bottle to prevent evaporation of the acetone, and is then quite stable.

A 1:10 dilution of the blood is made in the white pipette, and the mixture is thoroughly shaken three to five minutes. After blowing out the contents of the capillary tube, a drop is placed in the counting chamber (ruled for leukocyte count, i. e., 9 sq. mm.). Only the eosinophile cells are well seen; they appear as small, pink bodies. With a magnification of 120 to 150 diameters they are readily seen. The entire nine square millimeters of the chamber are counted. Ordinarily 9 to 18 eosinophile cells are found in this area; this corresponds to about 100 to 200 eosino-

[1] Dunger, R. "Eine einfache Methode der Zählung der eosinophilen Leukocyten und der praktische Wert dieser Untersuchung." *München. med. Wchnschr.*, 1910, LVII, 1942.

philic leukocytes per c. mm. The calculation of the total number of cells is made in the way described for counting the leukocytes (p. 238). After a little practice an increase in the number of these cells is recognized at a glance. By making a leukocyte count in the usual manner simultaneously, the *percentage* of eosinophiles may be determined.

Counting the Blood Platelets

Several methods have been proposed for counting the platelets. The *indirect method* has given fair results, i. e., making a count of the erythrocytes in the usual way and at the same time determining the relative number of platelets as compared to the red cells in a fresh specimen of blood. The number of platelets is then calculated. *Direct methods* of counting platelets have been attempted; the only one which appears to give reliable results is that of Wright and Kinnicutt.

Method of Wright and Kinnicutt.[1]—*The diluting fluid*:

Solution 1:

"Brilliant cresyl blue"..........	1.0 gm.
Distilled water	300.0 c. c.

Dissolve. Keep on ice to prevent the growth of yeasts.

Solution 2:

Potassium cyanid	1.0 gm.
Distilled water	1,400.0 c. c.

Method.—"The blood is mixed with the diluting fluid in the proportion of 1:100 by means of the pipette used for

[1] Wright, J. H., and Kinnicutt, R. "A new method of counting the blood platelets for clinical purposes and some of the results obtained with it." *Jour. A. M. A.*, 1911, LVI, 1457.

counting red blood corpuscles, and the counting is done in the ordinary counting chamber with a high power dry objective. In order to render the platelets more clearly visible, the specially thin cover glass of Zeiss, with central excavation, is used (cover glass No. 146, Zeiss catalog[1]). The diluting fluid consists of *two parts* of the aqueous solution of 'brilliant cresyl blue' (solution 1), and *three parts* of the aqueous solution of potassium cyanid (solution 2). These two solutions must be kept in separate bottles and mixed and filtered immediately before using. Of course, the pipette should be well shaken after withdrawing the sample for counting. After the counting chamber is filled, it is left at rest for ten or fifteen minutes, in order that the blood platelets may settle to the bottom of the chamber and be more easily and accurately counted.

"The platelets appear as sharply outlined, round or oval or elongated, lilac-colored bodies, some of which form a part of small spheres or globules of hyalin, unstained substance.

"The red cells are decolorized and appear only as 'shadows,' so that they do not obscure the platelets. The nuclei of the white cells are stained a dark blue, the protoplasm light blue. If the technique is correct, there should be no precipitate in the preparation.

"The cresyl blue solution is permanent, but should be kept on ice in order to prevent the growth of yeasts. The cyanid solution should be made up at least every ten days. It is, of course, necessary that the solution be made from pure potassium cyanid, which has not undergone decomposition. As already stated, the two solutions must be mixed and filtered immediately before using, because after filtra-

[1] This special cover glass is, however, unnecessary, if one has the usual thin cover glass, which permits the use of the high power dry objective.

tion, if the mixture is allowed to stand exposed to the air for a short time, a precipitate will form in it. After the diluting fluid has been mixed with the blood in the pipette, however, no precipitate forms, and, as the platelets do not quickly break up in the mixture, the counting may be done after some hours, if necessary. For example, a count immediately after filling the pipette was 258,000 and another count from the same filling of the pipette made eighteen hours later was 253,000.

"A proper technique yields a remarkably even distribution of the platelets in the chamber. For all practical purposes, the counting of the platelets in 100 small squares is sufficient, but for greater accuracy all 400 small squares should be counted, or 200 small squares in each of two fillings of the chamber."

With their method Wright and Kinnicutt find that the platelet count of normal adults varies between 226,000 and 367,000 per cubic millimeter, the general average being 297,000.

HEMOGLOBIN DETERMINATIONS

Many methods for the determination of the amount or percentage of hemoglobin have been brought forward. For a description of all of them the reader is referred to the textbooks of hematology.

(1) **Tallqvist** devised a *color scale,* which has been widely used. It consists of a series of ten shades of red, intended to represent the color intensity of hemoglobin from 10 per cent. to 100 per cent. Each color is perforated. A drop of blood is collected on a filter paper, supplied in the book containing the scale, and, as soon as the gloss has disappeared from the drop, it is placed under the perforation in one of the red strips. It is moved until the

color of the drop of blood corresponds with one of the shades of red. This represents the hemoglobin percentage of the blood. With the Tallqvist scale it is possible, perhaps, to make a more accurate guess as to the percentage of hemoglobin than without it. It is well recognized that the scale is very inexact. In fact, the color scales in separate books do not always match. When the blood is hydremic, the plasma runs beyond the corpuscles, which are concentrated at the center of the drop, introducing an additional error in the very cases where more exact results are desirable. If a hemoglobin determination is indicated a little more time should be spent than is required with the Tallqvist scale, in order to obtain a result of some value.

(2) **Sahli's Hemometer.**—Sahli's hemometer is a modification of the old Gower instrument. It consists (Fig. 42) of one tube containing the standard solution and a second tube of the *same caliber* graduated from 0 to 140, each division representing 20 c. mm. The tubes are placed in a hard rubber stand, which has an opaque glass back. A pipette with a line representing 20 c. mm. is supplied with the instrument. The standard solution is one of acid hematin, prepared as follows:[1]

Blood	1 part
$\frac{N}{10}$ hydrochloric acid............	10 parts
Distilled water to................	50 parts
Mix and add—	
Glycerin	50 parts

The hemoglobin is converted into acid hematin, which

[1] Hastings, T. W. "The estimation of hemoglobin-content of blood with modern instruments." *Jour. A. M. A.*, 1907, XLVIII, 1749.

does not go into solution, but is in a very fine state of suspension. Therefore, the hematin settles slowly, when the instrument is not in use, and for this reason a glass pearl is placed in the tube to facilitate mixing the standard fluid, which should be done each time immediately before using. Sahli [1] obtains the blood for the standard solution

FIG. 42.—THE SAHLI HEMOMETER.

from young adult males having a high red cell count. This explains the fact that normal blood seldom shows more than 90 per cent. of hemoglobin with the Sahli hemometer, when a new instrument is employed. In the course of time the standard solution fades. If the tube is protected from the light when the instrument is not in use, however, it may be kept as long as two years or more without serious deterioration. It is well to check the standard solu-

[1] Sahli, H. "Diagnostic Methods." 1st Amer. Ed., Phila. & London, 1905, p. 620.

tion from time to time with several bloods of normal adults having 5,000,000 red cells. With such a count the hemoglobin percentage should be 100. If the reading of the hemometer is too high or too low, the percentage of error is noted and the readings are then corrected correspondingly.

It is more satisfactory to prepare the standard solution from blood with a 5,000,000 count, checking it with other similar bloods. By doing this the standard tube may be refilled, say, every six or twelve months, doing away with the necessity of corrections. The values obtained are then safe for use in determination of the color index.

It is very important that the standard tube and the graduated tube have the same diameter.[1] If unequal, it is clear that the results obtained will be without value.

Method.—The graduated tube is filled accurately to the mark 10 with tenth normal hydrochloric acid. The pipette is now filled with blood exactly to the line marked 20 c. mm. The blood is quickly discharged into the acid in the graduated tube, and the pipette is rinsed two or three times with the acid to remove that which adheres to the wall of the pipette. The graduated tube is immediately shaken to secure a uniform suspension of the blood before clotting will have begun. The blood quickly becomes dark brown in color from the conversion of the hemoglobin into acid hematin. The mixture of blood and acid is allowed to stand exactly one minute, and is then diluted with water, until its color matches that of the standard solution. Day-

[1] Tubes from uniform tubing have been made for several years for the writer by Eimer and Amend, Third Ave., New York City. The standard tube is made in the form of the usual test tube. When filled with the standard solution it may be sealed in the flame, though it is more convenient to use a paraffined cork, as in this way it may be refilled an indefinite number of times.

light or artificial light may be used, since the tubes contain the same substance.

When the graduated tube is inverted to secure thorough mixing, care should be exercised that none of the fluid adheres to the finger, for enough may be removed in this way to cause a considerable lowering of the reading. When comparing the colors, it is well to rotate the graduated tube until the lines on it are not visible. When the colors have been accurately matched, the instrument is set aside for a couple of minutes to allow the fluid in the graduated tube adhering to the wall to run down. The height of the column is then read. This gives the hemoglobin percentage, the color of the standard fluid being considered as 100 per cent.

In cases where the hemoglobin is extremely low it is difficult to obtain satisfactory readings. In such case 40 c. mm. of blood may be added to the acid. The final result is then divided by 2.

Stäubli[1] has made a critical study of this method of determining hemoglobin, using the Sahli hemometer and the Autenrieth-Königsberger colorimeter. He finds that there is a progressive darkening of the acid hematin formed by mixing the blood with the tenth normal acid. The darkening is most rapid in the first few minutes after the mixture is prepared; plotting the values obtained, he found that the curve is a parabola. He has demonstrated that it is important to use tenth normal hydrochloric acid, not an approximate dilution, and to measure it into the graduated tube accurately, for the rapidity of darkening of the blood is directly proportional to the quantity and concen-

[1] Stäubli, C. "Zur Ausführung der Hämoglobinbestimmung. (Unter Umwandlung des Hämoglobins in salzsaures Hämatin.)" *München. med. Wchnschr.*, 1911, LVIII, 2429.

tration of acid. The blood-acid mixture should be allowed to stand exactly one minute, as Sahli recommends, and should then be quickly diluted with water, which inhibits the effect of the acid. Stäubli suggests that a better method of procedure with the Sahli hemometer is as follows: The blood-acid mixture is diluted at once with tenth normal hydrochloric acid, until the color is approximately that of the standard tube; then wait for ten minutes [1] to make the final comparison. The final dilution, which will require only a few drops, may be made either with water or with the acid. This technique in his hands has yielded uniform results with all bloods.

Whichever method is followed, it is absolutely essential that it be adhered to strictly in order to obtain comparable results.

Aside from variations in the standard fluids and possible lack of uniformity in diameter of the tubes, it is probable that Stäubli's findings explain to a great extent the anomalous results which many workers have obtained with this instrument.

Cleaning the Hemometer.—The graduated tube is rinsed with water. The pipette is rinsed first with water, then with alcohol and with ether. Finally, air is aspirated through it to dry the pipette.

(3) **The Fleischl-Miescher Hemoglobinometer.**—This instrument is generally considered to be the most accurate for the determination of hemoglobin. It is not well adapted to general use, since it is expensive and requires a dark room for making the readings.

The instrument (Fig. 43) consists of a standard, on which a wedge of red glass is mounted, cells of 12 and 15

[1] Ten minutes is the time interval selected, since it was found that the darkening which occurs beyond this interval is slight.

mm. depth, and a mixing pipette. The *pipette* is similar to those used with the hemocytometer. The markings on its capillary tube, ½, ⅔, and 1, permit of dilutions of the blood of 1:400, 1:300, and 1:200 respectively. Sodium carbonate, 0.1 per cent. aqueous solution, is used as the *diluting fluid*. The *cells*, 12 and 15 mm. deep respectively, are

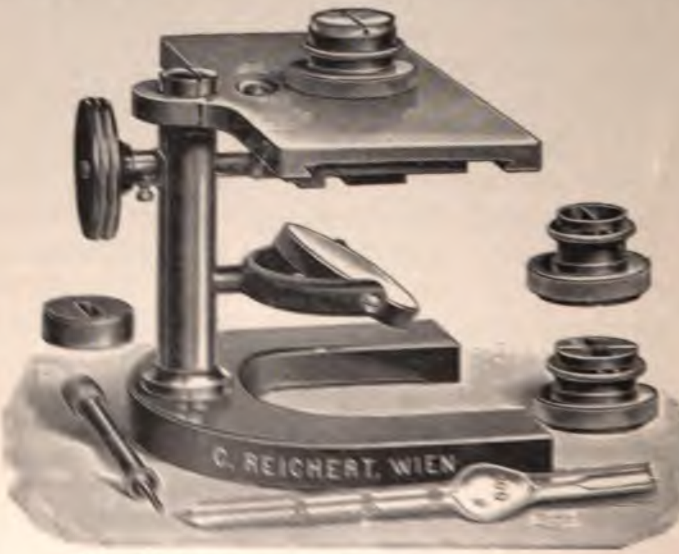


Fig. 43.—The Fleischl-Miescher Hemoglobinometer.

divided into two equal parts, one of which is filled with water, the other with the diluted blood. Each compartment should be filled until the surface of the fluid is convex above the upper level of the cell. The cell is then sealed by a glass disc, care being exercised to avoid bubbles in the fluids. Finally, a metal cup is placed over the glass disc. In the cup there is a slit, which should be so placed that its long axis is at a right angle to the partition dividing the cell. The cell is now placed on the stand, so that the compartment containing water is directly above the red

glass wedge. Candle light furnishes the most satisfactory illumination; it must be used in a dark room. All direct rays of light are cut off from the eye of the examiner, either with a large cone or by placing the instrument in a box with one side—at which the operator stands—open and a small hole cut in the opposite side near the bottom for illumination of the reflector. The colored prism is now moved until the shades of red in the two divisions of the cell are alike. The reading is made on the scale and recorded. Ten such readings should be made, and the average of them taken. If the cell 15 mm. deep has been used, the glass disc is removed and the diluted blood sucked back into the pipette. The 12-mm. cell is then filled, and ten readings are made with it, and the average taken. The latter should be four-fifths of the reading obtained with the 15-mm. cell, and should not vary by more than 2 per cent. The readings obtained do *not* represent hemoglobin percentages. They are to be used in connection with the table found in a pamphlet supplied with each instrument. (As the instruments are separately standardized, the tables often differ, and, therefore, cannot be used interchangeably.) From the table the hemoglobin in *grams per 100 c. c. of blood* is calculated according to the directions in the pamphlet. All values are to be reduced to a dilution of 1:300 with the 15-mm. cell. (For normal blood the 1:300 dilution is usually employed. With anemic blood use a dilution of 1:200, and with plethoric 1:400.)

The normal hemoglobin value with the Fleischl-Miescher apparatus is subject to considerable variation. Emerson [1] finds that in normal young adults the mean hemoglobin per 1,000,000 cells is 2.63 gm.

In leukemia or with extreme leukocytoses readings may

[1] Emerson, C. P. "Clinical Diagnosis." 1st Ed., pp. 466 *et seq.*

be difficult, because of the opacity produced by the white cells. The leukocytes may be removed by centrifugalizing the diluted blood before filling the cell.

Cleaning the Hemoglobinometer.—The pipette is cleaned in the same manner as the counting pipettes (q. v.). The cells should be taken apart, washed with water, dried, and reassembled.

(4) **Haldane's Hemoglobinometer.**—Haldane's hemoglobinometer is a very satisfactory instrument. Its only drawback—a minor one—is that illuminating gas is required in its use. Like the Sahli hemometer, it is a modification of the original Gower apparatus.

(5) **Dare's Hemoglobinometer.**—Dare's hemoglobinometer gives excellent results, but is fragile and expensive.

Sulphhemoglobinemia,[1] **Methemoglobinemia.**—The recognition of these abnormal pigments in the blood is described as follows by Clarke and Curts:[1] The blood is drawn from a vein, or, if this is not allowed, a few drops from the finger or ear will usually suffice. It is immediately diluted with twice its volume of distilled water, before clotting has taken place, and is thoroughly shaken. After the fibrin has separated, the solution is filtered several times through one filter paper, and the clear solution looked at through the spectroscope. The solution is then diluted drop by drop with water, until the red color (of the spectrum) stands out clearly. If there is a black absorption band in the red, either methemoglobin or sulphhemoglobin is present. If such a band persists after the addition of a drop of dilute ammonium sulphid, the pigment is sulphhemoglobin; if it disappears, it is methemoglobin.

In the blood the two bands of oxyhemoglobin are always

[1] Clarke, T. W., and Curts, R. M. "Sulphhemoglobinemia, with a report of the first case in America." *Med. Record,* 1910, LXXVIII, 987.

visible. In addition to these sulphhemoglobin presents a band in the red (near the orange) midway between C and D. With methemoglobin the band is again in the red, but nearer to C. (Compare with Fig. 7.)

COLOR INDEX

The color index is the quotient obtained by dividing the percentage of hemoglobin by the percentage of red corpuscles, 5,000,000 cells per 1 c. mm. being considered as 100 per cent. of corpuscles. Normally, the color index is about 1. When the index is less than 1 it indicates that the average corpuscle is poor in coloring matter, whereas with a high index the corpuscles are abnormally rich in hemoglobin.

VOLUME INDEX

The volume index of the blood was first studied by Capps.[1] He introduced the term to designate the quotient of the percentage volume of the erythrocytes divided by the percentage number of these cells.

Method.—To determine the volume of the red corpuscles, the *hematokrit* is employed. The usual form of apparatus is a hand or electric centrifuge armed with a frame for carrying two capillary tubes. The tubes are graduated from 0 to 100. Of the various procedures which have been proposed, Capps recommends the following: The capillary tube is completely filled with blood, the distal end of the tube smeared with vaselin, and placed in the carrier of the hematokrit. "Two conditions are essential to prevent coagulation, viz., scrupulous cleanliness of the tubes and speed in operation. The latter condition requires that the

[1] Capps, J. A. "A study of volume index. Observations upon the volume of erythrocytes in various disease conditions." *Jour. Med. Research*, 1903, X, 367.

blood must be placed in the hematokrit within a few seconds of withdrawal. . . . It is desirable always to fill two tubes as a control of one's results. The machine should be operated for three minutes at a uniform speed of ten thousand revolutions a minute" (Capps). The tubes are now examined, and it is seen that the corpuscles have been thrown to the distal end, leaving the clear serum proximally. With normal blood and a count of 5,000,000, the red corpuscles extend to the line marked 50, occupying one-half the capillary tube. This is the normal, and represents 100 per cent. volume. The erythrocytes are counted at the same time that the volume determination is made. The volume index $= \frac{\text{volume per cent.}}{\text{number per cent.}}$, 5,000,000 corpuscles being considered 100 per cent. In normal blood the volume index is 1. Owing to variations in the size of the erythrocytes in anemias, the percentage volume does not run parallel to the percentage number, as a rule.[1] The volume index, then, expresses the relative volume of the average red cell as compared with the normal.

In determining the volume of the red corpuscles, the leukocytes separate as a paler, grayish layer above the erythrocytes. Where their number is greatly increased, as in leukemia, determination of the volume of the red corpuscles is impossible.

The hematokrit furnishes a ready means of making macroscopic examination of the *blood serum*. Lipemia, cholemia, and hemoglobinemia may be revealed in this manner, if sufficiently marked, though hemoglobinemia may be an artefact from mechanical injury to the red corpuscles.

Cleaning the Hematokrit Tubes.—Blood should be

[1] Capps (*loc. cit.*) reports extremely interesting observations on volume index compared with color index and with measurements of the erythrocytes in primary and secondary anemias.

blown out of the tubes as soon as the reading has been made. The tubes are cleaned by drawing water, alcohol, and ether through them successively. If they are not perfectly cleaned, use acetic acid first, then the other fluids in the order given.

MEASURING THE DIAMETER OF CELLS

The diameter or a dimension of microscopic objects is expressed in micra (designated by the Greek letter μ), one micron being the thousandth part of a millimeter (0.001 mm.). In making measurements an *ocular micrometer* is employed. This is a glass disc, on which fifty equal divisions are marked by parallel lines. The upper lens of the eye-piece is unscrewed, and the micrometer is inserted in the tube of the ocular.[1] The value of the divisions on the micrometer scale is now determined in the following manner: The magnification of the microscope is varied by three factors, namely, the objective, the ocular, and the tube length. The usual tube length employed is 160 mm. Using this, the value of the spaces on the micrometer is determined with the objective and ocular to be used by comparison with an object of known dimensions. The most convenient object for this purpose is the counting chamber. The ruled area is placed under the microscope, and the number of divisions of the micrometer scale, which fall between the opposite sides of one of the smallest squares, or, in the case of low magnifications, between the sides of a larger unit, is found. Knowing the dimensions of the ruled surface, it is a simple calculation to compute the

[1] In ordering an ocular micrometer, the name of the maker of the microscope should be given, as the micrometer of one make may not fit the ocular of another.

value of a single division of the micrometer scale. The smallest squares are $\frac{1}{20}$ mm. on a side, or 50 micra.

As applied to the blood, measurements are usually made on stained films. At least one hundred cells should be measured, and, where much anisocytosis exists, two hundred cells should be the minimal number.

In the measurement of oval bodies, such as the eggs of many parasites, the two dimensions are readily obtained by rotating the ocular through ninety degrees.

VISCOSITY OF THE BLOOD AND OTHER FLUIDS

For clinical use, a number of instruments for determining viscosity have been described. That of Hess[1] has proved very satisfactory. It is compact and easily portable. The determinations may be made with a little practice in two or three minutes. The subject of viscosity is well discussed by Austrian.[2] The viscosity is compared with that of water.

Method of Hess.—The Hess viscosimeter (Fig. 44) consists of an opaque glass plate (H) on which two graduated tubes, A and B, are mounted. At one end these tubes communicate with a T-tube, G, which in turn is connected by rubber tubing with the rubber bulb L. At the other end the graduated tubes connect with capillaries C and D. The latter open into tubes E and F, which have the same diameter as the graduated tubes A and B. Capillary tube C and tube E are made in one piece, while tube F is held in apposition with tube D by means of the clip N. It is removable, and a number of similar tubes are supplied with the

[1] Hess, W. "Ein neuer Apparat zur Bestimmung der Viskosität des Blutes." *Münchcn. med. Wchnschr.*, 1907, LIV, 1590.

[2] Austrian, C. R. "The viscosity of the blood in health and disease." *Johns Hopkins Hosp. Bull.*, 1911, XXII, 9.

instrument. Through the valve Q it is possible to shut off the communication of the graduated tube B with the T-tube, and, therefore, with the rubber bulb as well. Between the rubber bulb and the rubber tubing a short piece of glass tubing is inserted; in it a hole is blown. This is opened or closed with the finger, and permits instant release of the negative pressure produced by the suction of the bulb. A thermometer is mounted on the glass plate.

Method.—With a pipette, which is furnished with the

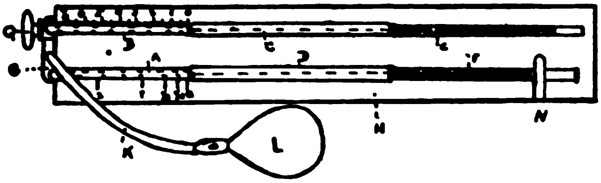


FIG. 44.—THE VISCOSIMETER OF HESS. (After Austrian.)

instrument, distilled water is placed at the opening of tube E. The valve Q is opened, and by suction from the bulb L the water is drawn into the tube E, until it reaches the capillary tube C. The pipette is then withdrawn, and the column of water is sucked further, until it reaches the mark 0 on the scale of the graduated tube A. The valve Q is then closed, the pressure having been released by removing the finger from the opening. (It is unnecessary to refill the tubes A, C, E, with distilled water for each determination; the water may be allowed to remain in the tubes and used repeatedly.) The tube F is then touched to a fresh drop of the blood to be examined. The blood should enter at the pointed end of the tube. When the latter is about three-fourths full the tube is held so that the blood will run down to the funnel-shaped end of the tube, which is then placed in contact with the free end of the

capillary tube D, and held in position by the clip N. By suction with the bulb the column of blood is then drawn to the line 0 on the scale of the graduated tube B, when the pressure is again released. The valve Q is now opened, and by suction through the bulb the column of blood is drawn to the mark 1 on the scale. It is drawn exactly to the mark, when the pressure is removed by withdrawing the finger from the opening. The point on the scale to which the water has been drawn represents the degree of viscosity of the blood. The viscosity of the blood of normal adults is about 4.55 (Austrian). If the viscosity of the blood is very great, or if the blood coagulates rapidly, the column of blood is drawn to the mark $\frac{1}{4}$ or $\frac{1}{2}$, and the result obtained is multiplied by 4 or 2 respectively. The error arising from making the observations at ordinary room temperatures is negligible (Austrian).

Cleaning the Viscosimeter.—As soon as the reading is made, positive pressure is exerted to expel the fluids from the graduated tubes. When the water reaches the zero line the valve Q is closed. The tube F is removed and the blood which escapes from the capillary tube D is caught on filter paper or cloth. A second tube, filled with concentrated ammonium hydrate, is placed in the clip, and ammonia is drawn through the tubes A, D, at least 2 cm. beyond the line 1 to which the blood extended. The ammonia is expelled and the tubes are refilled with fresh ammonia, which is allowed to remain in the tubes until the instrument is used again. The end of the capillary tube D is closed with a rubber cap. Immediately before using the apparatus the cap is removed and the ammonia expelled. If the pressure used to expel the ammonia is slight, only a trace remains, which is without appreciable effect on the result. It is essential that the tubes be perfectly clean.

If the apparatus is unused for some time, difficulty may be experienced in forcing the ammonia out of the tubes. This is usually due to the formation of ammonium salts at the opening of the capillary tube; they may be removed by solution in water. The valve should be lubricated with vaselin. The tubes F, after use, may be cleaned by aspirating water through them and then placing them in nitric acid for several hours. They are then dried by successive rinsings with water, alcohol, and ether. Erroneous results may be obtained if the tubes are dirty. The instrument should be tested from time to time with distilled water. If the result is not 1, the tubes are to be cleaned by drawing nitric acid into them. After an hour or so the acid is removed, the tubes rinsed twice with water, and then with ammonia.

THE SPECIFIC GRAVITY OF THE BLOOD

In clinical work the method usually used for determination of the specific gravity of the blood is that of Hammerschlag. A mixture of chloroform (sp. gr. 1.485) and benzol (sp. gr. 0.88) is placed in a cylinder. The specific gravity of the mixture should approximate that of normal blood (1.050 to 1.062). A capillary tube is filled with blood, which is flowing freely from the puncture wound, and a drop is allowed to fall into the mixture. If the drop sinks, its specific gravity is greater than that of the mixture, and more chloroform is added; if the reverse holds good, benzol is added. After each addition of chloroform or benzol the contents of the cylinder are well stirred. When a mixture is finally obtained in which the drop of blood neither sinks nor rises, its specific gravity is determined with an areometer. The result is approximately the specific gravity of the blood.

As Naegeli suggests, a series of mixtures of varying specific gravity is a great convenience.

The blood should not be permitted to drop into the chloroform-benzol mixture from a height, as it scatters. A bubble in the drop of blood may lead to serious error. Quick work is necessary to prevent the extraction of much water from the blood, and also to avoid evaporation of the mixture. After use the mixture may be filtered and kept in a brown glass bottle.

The *specific gravity of blood plasma or serum* may be determined by the method of Hammerschlag. Normally it lies between 1.029 and 1.032.

For the more accurate and time-consuming methods of determining specific gravity the reader is referred to works on hematology.

THE COAGULATION TIME OF THE BLOOD

The methods of determining the coagulation time of the blood are many, and the results obtained with each are more or less divergent. No perfectly satisfactory method has been brought forward. Among the best is that which employs the apparatus of Brodie and Russell, as modified and improved by Boggs.[1] Results almost as uniform have been published by Hinman and Sladen,[2] using their modification of Milian's method. An essential prerequisite to any method is absolute cleanliness of the apparatus and of the skin at the site of puncture.

[1] Boggs, T. R. "Some clinical aspects of blood coagulation." *Internat. Clinics*, 1908, I (18th series), 31.

[2] Hinman, F., and Sladen, F. J. "Measurement of the coagulation time of the blood and its application." *Johns Hopkins Hosp. Bull.*, 1907, XVIII, 207.

(1) **The Method of Brodie and Russell, as Modified by Boggs.**—The instrument (Fig. 45) consists of a moist chamber A and a truncated glass cone B, mounted to fit into the former. The lower surface of the cone is 4 mm. in diameter. Through the wall of the chamber a metal tube C extends, the tip of which approximates the lower surface of the cone. By means of a rubber bulb, such as is used on a camera, attached to the outer end of the metal tube, a current of air may be directed tangentially to the lower surface of the cone. The upper surface of the cone is covered by a cover glass D-E; at E there is a pinhole.

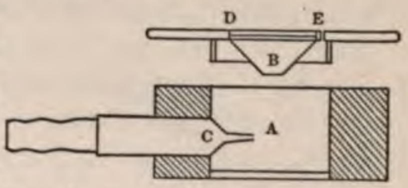


FIG. 45.—BOGGS' MODIFICATION OF THE COAGULOMETER OF BRODIE AND RUSSELL. A, moist chamber; B, cone of glass the lower surface of which holds the drop of blood; C, side tube, connecting with bulb; D and E, cover glass; at E, a pinhole. (After Emerson.)

Method.—A drop of blood is placed on the lower end of the glass cone, and the cone inserted in the chamber, which is then placed on the stage of the microscope. The drop is examined with the low power, and at the same time the bulb is squeezed, directing an air current against the periphery of the drop of blood. At first the corpuscles move freely in a circular direction. As clotting begins, masses of corpuscles take the place of the single cells. As coagulation progresses, the masses of corpuscles tend to become fixed in the drop. The air now displaces the masses in the direction of the current, but they spring back immediately after the air ceases to disturb them. The next stage, which is taken as the *end-point,* differs from the preceding in that a very gentle blast of air produces "radial elastic motion, as of a rubber ball pressed in at one point and released" (Fig. 46). When this point is reached the

time is again taken. The cone is immediately removed and the blood is wiped off on a cloth or filter paper to confirm the existence of a clot.

Occasionally a drop of blood fails to clot at one point. If this happens the result is valueless, and a second determination must be made.

The normal coagulation time of the blood with this instrument is between three and eight minutes, usually about five minutes (Hinman and Sladen).

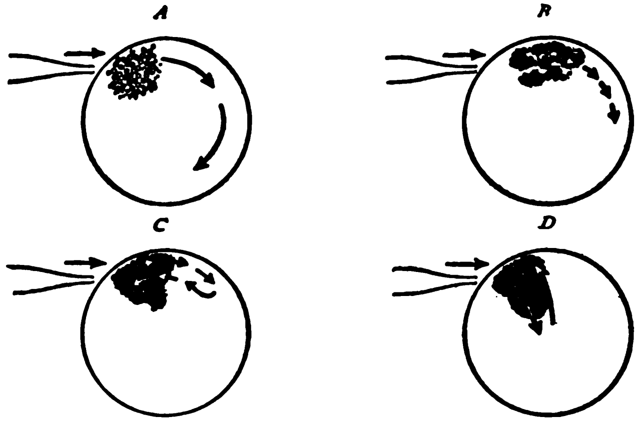


FIG. 46.—DIAGRAM TO ILLUSTRATE THE MOVEMENT OF THE CELLS DURING COAGULATION. D, the end-point. (After Emerson.)

Boggs emphasizes the following points of technique: The blood must be flowing freely from the puncture. When a sufficiently large drop has collected, the cone is touched to it at right angles to the surface of the drop and not dipped in it. In this way a drop of constant size is obtained. The coagulation time begins with the appearance of the drop of blood in the wound. Pressure upon the tissues and congestion of the parts are to be avoided, as they tend to increase the coagulability of the blood. Absolute cleanliness of the apparatus is essential. The air current should be gentle, and should not be applied too frequently.

(2) **Milian's Method, as Modified by Hinman and Sladen.**[1]—This method is extremely simple, requiring only clean glass slides and a millimeter scale. The ear is punctured, the first drop is discarded, and the time counted from the first appearance of the second drop. The lobule of the ear is held out, and the under-surface of the slide touched to the drop of blood, so that several small drops are obtained on it. The slide is then turned quickly to prevent the drops from flowing. Placing the slide over a scale, *only those drops having diameters of 4 and 5 mm. are allowed to remain,* others being wiped off. There are two methods of watching the drops to determine when coagulation has occurred; in each the slide is held vertically. In the one the profile of the drop is observed; before coagulation the drops sag, assuming the shape of a tear, while the uniform convexity is preserved after coagulation is complete. In the other method the vertical slide is examined by transmitted light. The denser portion will be found about the center of the drop, when coagulation has occurred; while the blood is still fluid the dependent part of the drop is the denser. The presence of a clot is then confirmed by transferring the drop to a cloth or filter paper.

Compared with Boggs' method, in which a 4-mm. cone is employed, the authors find that a 4-mm. drop clots more rapidly, a 5-mm. drop more slowly. They therefore take the mean coagulation time of several drops of 4 and 5 mm. diameter, respectively. The majority of records fall between five and eight minutes. Below eight minutes a record is within the limits for a normal coagulation time.

[1] Hinman, F., and Sladen, F. J. "Measurement of the coagulation time of the blood and its application." *Johns Hopkins Hosp. Bull.*, 1907, XVIII, 207.

Anything above eight minutes is delayed. When the coagulation time is delayed, only 5-mm. drops are used, in order to minimize the error due to evaporation.

THE RESISTANCE OF THE RED BLOOD CORPUSCLES

Numerous substances have been employed, against which the resistance of the red blood corpuscles has been measured. Solutions of sodium chlorid of varying strength, notably hypotonic solutions, have been most extensively used, and with them results of clinical importance have been obtained.

Method.[1]—Under aseptic precautions 2 to 5 c.c. of blood are aspirated from an arm vein, and immediately placed in five to ten times the volume of 1 per cent. sodium fluorid or 1.5 per cent. sodium citrate in 0.85 per cent. sodium chlorid to prevent clotting. As soon as the blood is discharged into the fluid, the flask is shaken well to insure thorough mixture. The blood-fluorid mixture is now centrifugalized at high speed to throw down the corpuscles. The supernatant fluid, containing the greater part of the blood plasma, is poured off. The plasma is then completely removed by washing the corpuscles three times in 0.85 per cent. solution of sodium chlorid. After the last washing the supernatant fluid is pipetted off, leaving the erythrocytes at the bottom of the centrifuge tube.

The hypotonic solutions of sodium chlorid diminish from 0.85 per cent. by 0.03 per cent., the solutions being 0.82 per cent., 0.79 per cent., and so on, down to 0.25 per cent. They are quickly prepared by filling one 50-c.c. burette, graduated to $\frac{1}{10}$ c.c., with distilled water, and

[1] Moss, W. L. "Paroxysmal hemoglobinuria: Blood studies in three cases." *Johns Hopkins Hosp. Bull.*, 1911, XXII, 238.

another with 1 per cent. aqueous solution of sodium chlorid. Thus, to prepare 10 c.c. of 0.70 per cent. sodium chlorid, take 7 c.c. of the 1 per cent. salt solution and 3 c.c. of distilled water.

A series of small test tubes is appropriately marked and placed in a rack. To each there are added 3 c.c. of hypotonic salt solution and 0.03 c.c. (about one drop) of the red blood corpuscles. The salt solution and blood corpuscles are well mixed by shaking. (The tubes are, of course, perfectly clean and sterile, and are plugged with cotton.) After all have been filled, they are placed in the ice chest to prevent bacterial growth, and are allowed to remain until the red cells have settled to the bottom. For the lower dilutions this usually requires about two hours. The supernatant fluid is now examined for free hemoglobin, the presence of which shows that there has been laking of the corpuscles.

The tube of lowest dilution showing even a trace of hemoglobin in the fluid represents the so-called *minimal resistance.* That is, with this strength of salt solution the least resistant cells are "laked," their hemoglobin escaping from the cell membrane into the salt solution. The *maximal resistance* is found by noting the strength of salt solution in which *all* the red corpuscles are laked.

Normally the minimal resistance, in terms of hypotonic salt solution, is about 0.47, the maximal resistance about 0.30.

THE EXAMINATION OF FRESH AND STAINED PREPARATIONS OF BLOOD

The first requisite in the preparation of fresh or dried films of blood is perfectly clean glassware.

The Cleaning of Cover Glasses and Slides.—Of the vari-

ous methods used to clean glassware for blood work in the author's laboratory, the following has given the most satisfactory results, and is always dependable:

(1) Immerse the covers (or slides) in concentrated sulphuric acid for about twenty-four hours.

(2) Pour off the acid and wash in running water.

(3) Drain off the water and cover the glassware with 95 per cent. alcohol for an hour or longer.

(4) Replace the alcohol with chloroform and dry the glassware as needed.

The covers should be dried with a perfectly clean cloth, free from lint. An old linen handkerchief which has been laundered many times is suitable. If the glassware is to be kept dry, it should be placed in a dust-proof receptacle.

Ether may be substituted for chloroform, but is less satisfactory.

For blood work ¾-in. square cover glasses, No. 1, are the best. The 3x1-in. glass slides should be thin, with straight, even edges, if they are to be employed in making blood films. If cover glasses are used in making the films, the finish of the slide is less important.

Examination of the Fresh Blood

In the examination of the fresh blood, a procedure which is too generally neglected, the specimen is prepared in the following manner: The ear is pierced, but the puncture should not be so deep as to cause a very free flow of blood, since it is essential to be able to regulate the size of the drop accurately. Therefore, a small, superficial puncture is made, from which the blood will escape easily on *very gentle* pressure. (Pressure is to be avoided as far as possible, to prevent the dilution of the blood with tis-

sue lymph. In grasping the ear the fingers should be at least ½ inch from the wound. It is better, of course, to obtain the blood without any pressure whatever.) A drop of blood about the size of a small pinhead is transferred to a cover slip, which is immediately placed upon a glass slide. The blood spreads out between the cover glass and slide in a thin film. Microscopic examination should show the individual red corpuscles separated from one another in the central portion of the film, with the thicker parts at the periphery presenting rouleaux formation. If the cells are not separated the drop of blood used was too large, provided the glassware was clean and the drop of blood fresh.

Failure to obtain satisfactory specimens is usually attributable to one of several causes. If the drop of blood is allowed to remain on the ear an appreciable length of time before it is used, clotting may have begun; this, of course, interferes with the proper spreading of the blood. Again, any dirt on the ear also has the same effect. Particles of dust or bits of lint on the glassware prevent even uniform spreading of the blood by elevating the cover glass from the slide. As dust frequently settles on the cover glasses or slides while preparing to secure the blood, it is a good plan to remove all such particles by blowing on the glass (avoid moisture from the breath on the glass), or by brushing the surfaces with a camel's hair brush. Any grease or dirt of any kind on the glass makes it impossible to obtain good specimens. It is advisable to handle the cover glasses with a pair of straight forceps, to avoid the grease, etc., of the fingers.

Sealing the Fresh Specimen.—If the specimen is to be kept for any length of time, it should be sealed to prevent drying. Vaselin is convenient for this purpose. A small

quantity of it is taken up on the end of a match, which is then rapidly passed through a Bunsen flame. The edge of the cover glass is now lined with the melted vaselin, which hardens almost instantaneously, and effectually seals the specimen. Paraffin of low melting point may also be used. Specimens prepared in this way may be kept for a surprising length of time with little alteration in the red corpuscles.

The Preparation of Dry (Permanent) Blood Smears.—(1) *The Cover Glass-Forceps Method.*—In the writer's experience the best results are obtained by using two cover glasses. The covers are cleaned and dried as described on page 265. Any particles of dust are carefully removed from the covers just prior to making the smear. Forceps are used to avoid soiling the surfaces of the cover glasses with the fingers. With care, however, perfectly satisfactory films may be made with the fingers.

Two pairs of forceps are needed. One is a *cross-billed forceps,* which will hold a cover glass firmly. The spring should be strong and the blades perfectly parallel, so that the grip on the cover slip will be uniform. If the forceps are suitable it should be possible to lift them by grasping a cover glass caught between the blades of the forceps without changing the relative position of the cover glass. Forceps which cannot withstand this simple test usually prove to be useless. A pair of *straight forceps* is also required. They should be fairly stiff, with blades having plain, square ends. When holding a cover slip firmly ***only the tips*** of the blades should touch it.

To prepare blood films a clean cover glass is placed in the cross-billed forceps, the puncture wound is then wiped free of blood, and, when a drop of the proper size appears (about the size of a small pinhead with a normal count,

larger with anemic blood), it is taken up on a second cover slip held in the straight forceps. This is immediately placed on the first cover glass. The blood spreads out between the two in a thin layer. Just *before* the drop will have stopped spreading between the covers, the overlapping edge of the second cover is grasped with the straight forceps, and the two are quickly pulled apart. It requires considerable practice to pull the covers apart in exactly parallel planes, which is necessary if the spreads are to be good. With good preparations microscopic examination will show the individual red cells well separated over one-half to two-thirds of the preparation. With a little experience good smears may be selected with the unaided eye. When inspected by transmitted light, the area in which the cells are properly separated resembles an extremely thin, gray veil; if the cells are grouped in little islands, the uniformity of the veil is lost. The thick parts of the smear are more dense and opaque.

The films, which are allowed to dry in the air, are then ready for fixing and staining. At times, when the humidity is very high, it may be necessary to fan the films to hasten the drying. (During the fly season films should be protected from the pests, as they may eat practically all the blood from a cover glass in a few seconds.)

The size of the drop of blood is a matter of great importance in making blood smears with the cover glass method, as has been indicated above. The correct size will depend largely on the number of red corpuscles in the blood. With very anemic patients, whose blood is thin and hydremic, a relatively large drop will be needed. The general tendency of beginners is to take a drop which is too large. If this mistake is made, no part of the film is thinly spread, the erythrocytes being piled up so that

study of the individual cells is impossible. If one waits until the blood has stopped spreading, it is often impossible to separate the covers, as they become sealed. Lint, dust, gritty particles, or grease on the cover glasses will make it impossible to secure satisfactory specimens.

(2) *The Glass Slide Method.*—Many clinicians prefer glass slides to cover glasses in making blood films.[1] The method requires practically no practice, and is simpler than the cover glass-forceps method. The area of the blood film may be made much larger than that obtainable on a cover glass. The slides should be thin, and should have perfectly smooth, even edges and level surfaces. They must, as a matter of course, be perfectly clean. Any dust which may have settled on the slides should be removed before using them.

A drop of blood considerably larger than that required in the cover glass method is taken up on the end of one slide, which is then approximated to the surface of a second slide, placed on a table or other firm surface. The first slide is held at an angle of about 45 degrees to the second. The blood spreads out along the end of the first slide, which is now *pushed* rather rapidly along the surface of the second slide. The blood spreads out in a thin layer over the surface of the second slide. In making the spread, pressure on the slides is unnecessary.

[1] In the experience of the author, more satisfactory specimens are obtained with the cover glass method. As a rule, the leukocytes are more evenly distributed over the specimen. The large smear, which is obtained with the slide, is usually no advantage, for it is seldom the case that a greater area is needed than is contained in a cover glass preparation. The great value of the slide method, aside from the fact that good smears may be obtained with it, lies in the fact that the technique is easily acquired, and fair specimens may often be obtained with slides which have been cleaned only with water. All laboratory workers should, therefore, be able to employ the method, though the cover glass method is preferred.

Labeling the Blood Films.—With specimens made on glass slides, where the area of the blood film is large, a part of it may be employed for labeling the specimen. A very simple and practical method has been described by von Ezdorf.[1] The necessary data is written on the thick part of the film with a soft, black lead pencil. The label thus made is permanent, and is not affected by staining or washing the specimen. The black contrasts well with the usual pink color of the film.

Fixation of Blood Smears.—Various methods are available for fixing the blood cells to the slide. The following will be found useful:

(1) *Heat Fixation.*—A triangular copper bar, first introduced into blood work by Ehrlich, is usually used for heat fixation. The bar is placed on a tripod with a Bunsen flame under the tip of the bar. In a short time, if protected from strong drafts, all parts of the bar acquire and maintain a fairly constant temperature. By dropping water from a pipette onto the bar, the point *farthest from the flame* is determined, at which the drop of water remains spheroidal and rolls off. The temperature at this point, the "spheroidal point" for water, is about 150° C. The point is marked, and the blood films, with the specimen side up, are then placed just inside this point, i. e., toward the flame from the spheroidal point, and allowed to remain 30 to 45 seconds. This usually suffices to fix the films well. In certain instances a longer or shorter time is required, the extremes falling between 5 and 120 seconds. By placing four specimens of blood (cover glass preparations) at the spheroidal point and removing them at the end of 30, 35, 40, and 45 seconds respectively, and staining all, the proper

[1] Von Ezdorf, R. H. "The labeling of dried blood films." *Jour. A. M. A.*, 1910, LIV, 125.

fixation time is quickly determined with the great majority of bloods. It is to be remembered that there is no one optimal fixation time applicable to all bloods. A separate determination must be made for each individual blood examined.

In place of the copper bar, an *oven* may be used. The specimens are placed in the oven, which is maintained at a temperature ranging between 110° and 120° C. for one to two hours, rarely longer. By removing and staining a specimen every fifteen minutes after the first hour, the correct fixation time is determined. This method of employing heat fixation is particularly convenient, when a large number of specimens of the same blood are to be fixed.

Heat fixation is always used with Ehrlich's triacid stain. In using Pappenheim's methyl green-pyronin mixture, heat fixation is also to be preferred. It is less useful for other blood stains.

(2) *Ethyl Alcohol.*—The specimens may be fixed by immersion in absolute alcohol one to five minutes, or in 96 per cent. alcohol five to twenty minutes. They are then dried in the air or between blotting paper. Less expensive and about as satisfactory is the denatured alcohol of commerce.

This method of fixation is useful in connection with hematoxylin and eosin, methylene blue, etc.

(3) *Methyl Alcohol.*—Absolute methyl alcohol, acting for one to five minutes, is an excellent fixative. (Methyl alcohol fixation is carried out as a part of the staining technique in employing Leishman's, Wilson's, and Jenner's stains, as will appear below.)

Methyl alcohol may be used in place of ethyl alcohol, and is usually employed with Giemsa's stain.

(4) *Acetone.*—The specimens are placed in acetone five minutes, and are then dried in the air.

(5) *Alcohol-Formalin.*—Futcher and Lazear have used 0.25 per cent. formalin in 95 per cent. alcohol. The solution must be prepared freshly, and is obtained by adding one drop of commercial formalin (40 per cent.) to 10 c. c. of alcohol. The specimen is allowed to remain in this mixture one minute, and is then washed in water and blotted dry.

This method is the best for staining with carbol-thionin.

Staining the Blood

The stains and combinations of stain for blood work are numerous. Those described below are among the most serviceable, and enable one to make all routine examinations. Most of the stains are applied to dried, fixed films of blood. The staining of the fresh, unfixed blood, the so-called "vital" staining of the blood, forms an exception.

"Vital" Staining of the Blood

Various stains and methods have been proposed for vital staining of the blood. Practically any basic dye may be used for this purpose, but the stains which have been used most extensively are Unna's polychrome methylene blue (Grübler's), Pappenheim's methyl green-pyronin, and neutral red. Only the first is described, since the picture obtained is rather more brilliant.

(1) **Vaughan's**[1] **Method.**—A small puncture is made in the ear, and over the wound, from which the blood has been wiped, a minute drop of Unna's polychrome blue is

[1] Vaughan, V. C., Jr. "On the appearance and significance of certain granules in the erythrocytes of man." *Jour. Med. Research*, 1903-4, X, 342.

placed by means of a clean glass rod. A small drop of blood is now pressed out of the wound, so that it flows directly into the stain. The procedure is now the same as in the preparation of a specimen of fresh blood (q. v.). The relative proportions of stain and blood are quickly learned by experiment. There should be more blood than stain in the mixture. After the specimen has spread out between cover glass and slide, it is ready for examination. It should be sealed, if the examination is to be a prolonged one.

On microscopic examination with the oil immersion objective, the majority of the red corpuscles appear quite like those in a preparation of fresh blood, except where the stain is concentrated; here the cells may show a diffuse purplish tint of varying intensity. Laking may occur in a certain number of the corpuscles. In some of the red cells granules stained bluish-purple are seen. These basophilic substances have been designated "granulo-reticulo-filamentous" by Sabrazès.[1] There may be very few granules in a cell, or they may be extremely numerous. Often the granules appear to be attached to a delicate filament, which may form a part of a reticulum in the corpuscle. Not infrequently the granules are clustered at the center of the cell, suggesting by their position and number the remnants of a nucleus. In erythroblasts the nucleus takes a purple color, as do nuclear particles, when present. The chromatin of the blood platelets takes on a similar color, and may often be seen occupying a position at the periphery of a clear, unstained globule, as Vaughan observed. Leukocytic nuclei stain more or less intensely, depending largely

[1] Sabrazès, J., and Leuret, E. "Hématies granuleuses et polychromatophilie dans l'ictère des nouveaux-nès." *Gaz. hébd. d. soc. méd. de Bordeaux*, 1908, XXIX, 123.

on the concentration of the stain. In the protoplasm of the polynuclear cells, stained granules may be found. The ameboid leukocytes retain their activity for some time. The colorless cell membrane may be seen extending some distance beyond the granules in the pseudopods of the neutrophilic cells.

(2) **Method of Widal, Abrami, and Brulé.**[1]—"Four to six drops of blood are allowed to fall into a test tube containing 10 drops of a basic coloring matter, which is quite isotonic, and contains in addition oxalate of potassium to prevent the coagulation of the blood.

Potassium oxalate, 20 per cent. solution	2.0 c.c.	$\Delta = -0.60$
Unna's polychrome methylene blue	100 drops	

"The fresh corpuscles are allowed to remain for 10 to 20 minutes in contact with the solution, after which the mixture is centrifugalized, the supernatant fluid is removed, and the corpuscles drawn up with a pipette and placed upon slides, upon which they are spread as an ordinary drop of blood; the covers are then dried and fixed by heat. Such preparations may be preserved indefinitely" (Thayer and Morris).

(3) **The "Dry" Method of Vital Staining.**—The dry method of vital staining consists in spreading a thin film of stain on a glass slide, allowing it to dry in the air, protected from dust, and then placing a cover glass with a drop of blood on the dried stain, just as in making a preparation of the fresh blood. The blood spreads out between

[1] Widal, F., Abrami, P., and Brulé, M. "Diversité de types des hématies granuleuses; procédés de coloration." *Compt. rend. Soc. de biol.*, Par., 1908, LXIV, 496.

cover and slide, the stain dissolves in the plasma, and the result is much the same as with other methods. With this method there is less danger of laking the corpuscles.

Pappenheim's methyl green-pyronin mixture has been used extensively by the French, usually with the dry method.

Neutral red may be employed. A dilute solution of the dye is prepared in physiological salt solution, or a smaller quantity of saturated, aqueous solution of the stain may be used. It may be substituted for polychrome methylene blue in Vaughan's method, or may be used in the dry method.

In normal blood of adults less than 1 per cent. of the erythrocytes contain the granulo-reticulo-filamentous substance, while in newborn infants the number is 7 per cent. or less (Vaughan).

The Staining of Dried Blood Films

The stains which are required for the routine examination of blood are Ehrlich's triacid, Jenner's stain, and a Romanowsky stain. For special purposes, however, other stains are required at times.

(1) **Methylene Blue.**—(1) Fix the blood film in alcohol.

(2) Stain with Löffler's methylene blue (p. 213) about 3 to 5 seconds.

(3) Wash in water, blot dry, and mount in balsam.

The stain is useful as a nuclear stain. For the demonstration of basophilic granules and polychromatophilia, methylene blue is one of the most reliable stains.

Nuclei are stained dark blue. The leukocytic granules are unstained, excepting basophilic granules, which take a

bluish-purple color. The basophilic protoplasm so frequently encountered in lymphocytes is stained a paler blue than the nucleus, the shade varying greatly in different cells. The *erythrocytes* assume a pale, greenish tint. Polychromatophilic red cells are light blue to very deep blue, depending on the degree of polychromatophilia. Basophilic granules in the red cells are stained dark blue, almost as dark as the nuclei. Nuclear particles in the red corpuscles take the same color as the nuclei of erythroblasts, i. e., a dark blue. *Blood platelets* are indistinct, appearing as dirty grayish-blue masses.

(2) **Eosin.**—Eosin may be used as a counter-stain in ½ per cent. aqueous solution. It is used after the methylene blue has been washed off the specimen. The stain is allowed to act a few seconds, the intensity of staining being controlled by microscopic examination of the film in water. Slower staining is secured by diluting the staining solution with water. Eosin adds little to the picture, except that it stains the eosinophilic granules, which now assume a brilliant pink or reddish-pink hue. However, slight polychromasia may be somewhat less evident, though often more striking because of the contrast. The orthochromatic erythrocytes are stained pink. If the specimen has been overstained with eosin, the pink color will be apparent in the protoplasm of the lymphocytes and neutrophilic leukocytes.

(3) **Hematoxylin.**—Ehrlich's acid hematoxylin is prepared as follows:

Solution A:

Hematoxylin	2.0 gm.
Alcohol, absolute	60.0 c. c.

Dissolve.

Solution B:

Saturated solution of alum in equal parts of glycerin and distilled water	60.0 c. c.
Glacial acetic acid	3.0 c. c.

The two solutions, A and B, are mixed and allowed to "ripen" in an open bottle for a week. The bottle is then stoppered. The ripened stain has a reddish-blue color. If the bottle is shaken or disturbed, the solution should be filtered before using.

Method.—(a) Fix the blood films in alcohol. Heat fixation may also be used.

(b) Stain in hematoxylin 2 to 10 minutes or longer. Control the intensity of staining by examining the specimen in water.

(c) Wash in tap water. The washing may be completed in a few seconds, but the beauty of the nuclear staining is greatly enhanced by prolonged washing in tap water. If the specimen has been overstained with hematoxylin, it may be cautiously decolorized in acid alcohol (HCl, 1.0 c. c., 70 per cent. alcohol, 100.0 c. c.), and again washed in water. It is better to avoid overstaining by controlling the staining carefully under the microscope.

(d) Dry, mount in balsam.

Hematoxylin is one of the best nuclear stains. For studying the morphology of nuclei it is particularly useful.

Nuclei are stained a very dark blue, at times almost black. After prolonged washing, however, the blue is brighter—more brilliant. As with other nuclear dyes, the color intensity in a given nucleus depends, of course, on the amount and concentration of the chromatin. Mast-cell granules are stained dark blue, but may be lost after wash-

ing the specimen. Other leukocytic granules are unstained. Basophilic protoplasm is less intensely stained than with methylene blue. The *red blood corpuscles* are lightly stained, and are either gray or grayish-blue. The more marked grades of polychromatophilia are revealed by the darker blue stain of the cells. Coarse basophilic granules in the erythrocytes are fairly well demonstrated as dark blue spots; the finer granules are unstained or indistinct, as a rule. Nuclear particles take on a very intense, dark blue, like the pyknotic nuclei of normoblasts. *Blood platelets* are dirty blue and indistinct.

Eosin may again be employed as a counterstain. When the specimen is properly stained with eosin, no pink is seen in the protoplasm of the neutrophilic cells, while the eosinophilic granules stand out prominently. Hematoxylin and eosin are useful in cases where the relative number of eosinophilic cells is to be determined, as a differential count with this point alone in view may be made rapidly.

(4) **Carbol-thionin.**

Saturated solution of thionin in 50 per cent. alcohol..............	10.0 c. c.
Carbolic acid, 1 per cent.........	100.0 c. c.

(a) Fix the blood films in alcohol-formalin.

(b) Stain with carbol-thionin $\frac{1}{4}$ to 3 minutes.

(c) Wash in water. If the specimen is overstained, the washing may be continued, or the specimen may be decolorized in 50 per cent. alcohol.

(d) Dry, and mount in balsam.

The stain is an excellent nuclear stain. All *nuclei* are stained dark blue. Leukocytic granules are not specifically stained with the exception of the granules of the mast cells,

which are purple. The *red blood corpuscles* are greenish-gray. Basophilic granules are dark blue, polychromatophilic red cells varying shades of blue. Nuclei and nuclear particles are dark blue. The bodies of malarial parasites are purple, contrasting well with the red blood corpuscles. The nuclei of the parasites are unstained. *Blood platelets* are indistinct, and have a mauve color.

Preparations stained with carbol-thionin fade in the course of several months, as a rule.

Staining Mixtures of Two or More Stains

(5) **Ehrlich's Triacid Stain.**—For the sharp differentiation of neutrophilic granules, the triacid stain of Ehrlich is unequaled. It should always be used in the study of these cells. The mixture contains three stains, two of which, orange G. and acid fuchsin, are acid, the third, methyl green 00, basic. The three basic radicals of the methyl green are satisfied by the acid dyes, hence the name, triacid. The formula [1] given below, a slight modification of the usual one, has been found to yield uniformly good staining mixtures,[2] whereas formerly it has been more or less a matter of good fortune to obtain a satisfactory solution. Good staining mixtures may usually be had from Grübler. In preparing the mixture, saturated aqueous so-

[1] Morris, R. S. "The value of Ehrlich's triacid stain in blood work." *Jour. A. M. A.*, 1910, LV, 501.

[2] Recently (1912) we have encountered the first failures in more than five years. After numerous experiments the cause of the trouble was found to lie in the acid fuchsin. Three different lots of the powdered stain in Grübler's original packages obtained through one firm resulted in poor staining mixtures, while acid fuchsin secured from another firm (also Grübler's make) gave very satisfactory results. With the poor acid fuchsin all cells were stained diffusely red. The nature of the defect in the acid fuchsin has not yet been determined.

lutions of methyl green,[1] orange G., and acid fuchsin are made separately. They must be allowed to settle for at least a week before use, and should be replenished as needed, so that a constant supply of the stock solutions may be on hand. Grübler's stains are generally used.

The formula, as modified, is:

Saturated aqueous solution of orange G......	13.0 c. c.
Saturated aqueous solution of acid fuchsin...	7.0 c. c.
Distilled water	15.0 c. c.
Absolute alcohol	15.0 c. c.
Saturated aqueous solution of methyl green..	17.5 c. c.
Absolute alcohol	10.0 c. c.
Glycerin	10.0 c. c.

The fluids are mixed with the same graduated cylinder, which should not be rinsed. The receiving flask should be shaken vigorously after the addition of each constituent, which is added in the order given in the formula. It is essential to add the methyl green, second portion of alcohol, and glycerin slowly, shaking well after each addition. The mixture is ready for use immediately, and does not deteriorate with age. After the mixture has stood a while a small amount of precipitate may form. Care should be exercised that this is not disturbed when using the stain.[2]

Method of Staining.—(a) Fix the blood spread by heat (p. 271).

(b) Stain 5 to 10 minutes (overstaining is impossible).

(c) Wash quickly in water, blot dry with filter paper, and mount in balsam.

In a properly fixed specimen the neutrophilic granules

[1] Methyl green is used in place of methyl green 00 of the original formula.

[2] For blood stains, bottles with droppers, the rubber nipples of which also serve as stoppers, are indispensable. Barnes' bottle is a very good one.

stand out sharply. When this is the case the erythrocytes are usually, though not always, colored deep orange or buff; in an underfixed specimen they are stained red, while too prolonged fixation causes them to take a yellow color. The color of the red corpuscles, while a safe index of the fixation in most instances, fails at times. The final criterion by which a specimen is judged must be the staining of the neutrophilic granules.

In a good specimen (plate I) the *erythrocytes* have, then, a buff color usually. Polychromasia is not demonstrated. Basophilic granules and Cabot's ring bodies are unstained. The pyknotic nuclei of normoblasts take a dark green color, the megaloblastic nuclei being less deeply stained. Often reddish areas are visible in the nuclei. Nuclear particles are stained green, but are much less striking than when stained with better nuclear stains, such as hematoxylin or a Romanowsky stain. Malarial and other parasites are not well stained. It is evident, therefore, that the triacid stain is very inferior for the study of pathological changes in the red blood corpuscles. Many of the abnormalities of the red cells are not shown at all, and none of them is as well demonstrated as with a Romanowsky stain or with Jenner's stain.

Blood platelets appear as ill-defined, indefinite, mauve-colored masses.

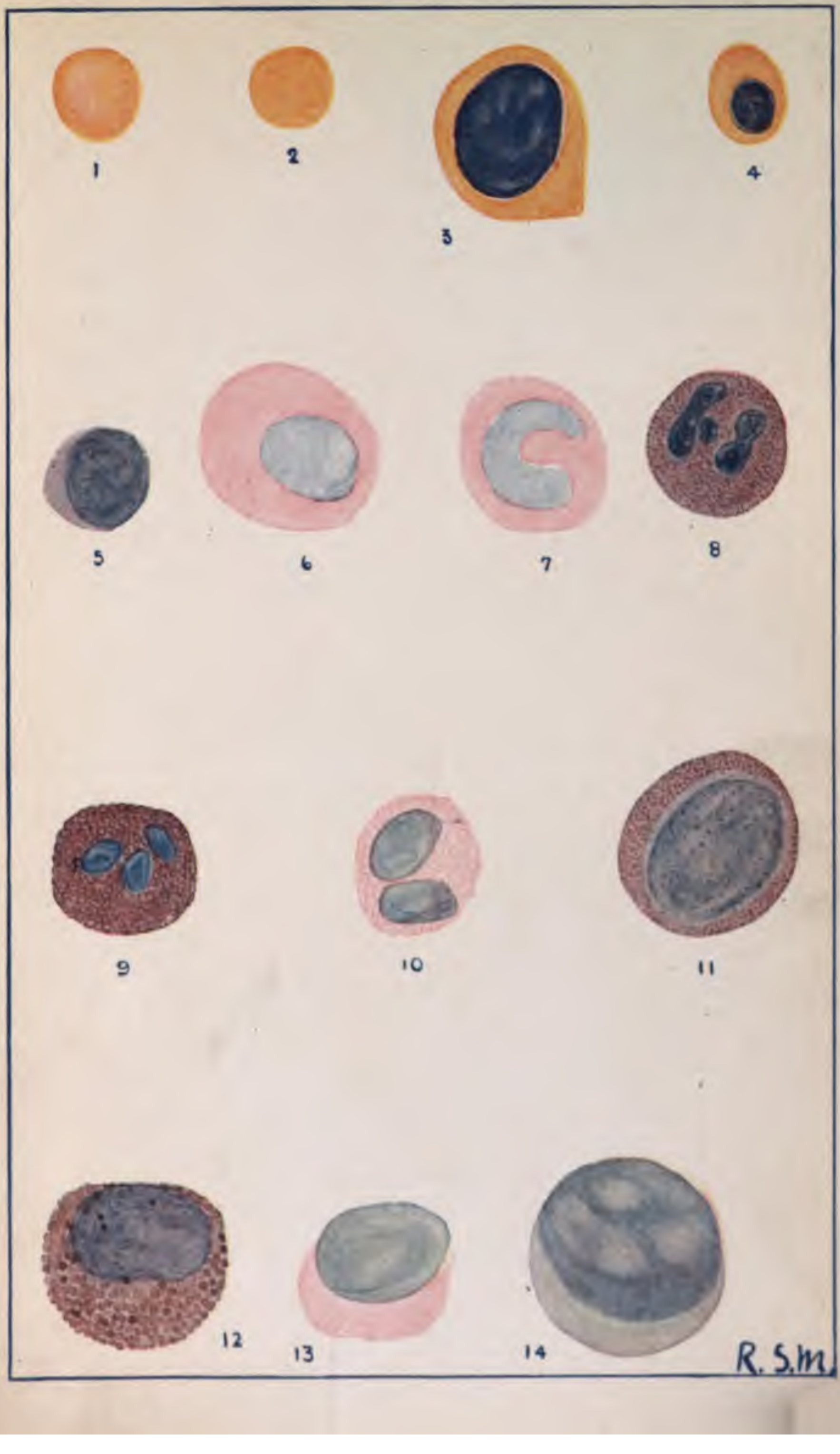
Leukocytic granules are well differentiated, but the *nuclei* are poorly stained, assuming a rather pale green or bluish-green color. All neutrophilic granules take a lilac color. There should be no blurring of them in a well-fixed specimen; the granules are sharply defined and distinct, though in the myelocytes the very fine granules stand out less prominently than in the polynuclear cells. Eosinophilic granules assume a brick-red or coppery color, while

LEGEND FOR PLATE I.

(All drawings made with camera lucida; × 1200. Ehrlich's triacid stain.)

1, 2. Normal red corpuscles.
3. Megaloblast.
4. Normoblast.
5. Lymphocyte.
6. Large mononuclear leukocyte.
7. "Transitional" leukocyte.
8. Polynuclear neutrophilic leukocyte.
9. Polynuclear eosinophilic leukocyte.
10. Mast cell or polynuclear basophilic leukocyte.
11. Neutrophilic myelocyte.
12. Eosinophilic myelocyte.
13. Mast myelocyte or basophilic myelocyte.
14. Myeloblast.

PLATE I



basophilic granules are unstained, appearing as vacuoles in the cytoplasm. The protoplasm of the lymphocytes is either colorless or a faint rose-pink. The same holds true for the large mononuclear and transitional cells; their nuclei, being poor in chromatin, usually stain very faintly, so that they are easily overlooked. Because of this difficulty with the non-granular leukocytes, Löffler's methylene blue has been used to stain the nuclei more intensely. It is applied to the blood film for a few seconds (3 to 5) after the staining with the triacid has been completed. The granular stain may be slightly impaired, but the nuclei are much more evident. The mast-cell granules are now stained purple. In this connection it may be added that Pappenheim has prepared a triacid mixture, substituting methylene blue for methyl green, but it has not been widely adopted.

(6) **The Romanowsky Stains.**—The Romanowsky stains are by far the best for the demonstration of pathological changes in the red corpuscles, and are, of course, indispensable in the study of such protozoa as the plasmodia of malaria, trypanosomes, etc.

Romanowsky's original method of preparing the stain has undergone numerous modifications, with a view to simplification both of the preparation of the stain and of the staining technique. The essential dyes are eosin and methylene azure, the latter being obtained from methylene blue. Methods of preparing the stain have been described by a number of workers in this country, among whom may be mentioned Wright, Harris, Hastings, MacNeal, Wilson. Leishman's stain, which antedates all of those mentioned, is used extensively in England. The staining mixtures may be purchased; it is much more convenient and satisfactory, however, in private laboratories of physicians

where only moderate amounts of stain are used, to buy tablets of the powdered stain. A tablet is dissolved in a stated quantity of absolute methyl alcohol (usually 10 c. c.), and the mixture is ready for use at once. In this way fresh stain may be had at frequent intervals, and there is less danger of deterioration of the mixture. Such tablets are prepared by Burroughs, Wellcome, & Co., and by Grübler. The method of preparation of only one of the modifications of the Romanowsky stain, Wilson's, is given. The writer has employed it for several years with entire satisfaction.

(a) WILSON'S STAIN.[1]—Prepare a 1 per cent. aqueous solution of methylene blue,[2] which contains 0.5 per cent. of sodium carbonate and at least 0.5 per cent. of freshly precipitated silver oxid.[3] The solution is boiled for twenty minutes; then remove one-third of it. After boiling another twenty minutes, remove one-half. Continue to boil the remaining portion twenty minutes. The three portions are now united and distilled water is added to the original volume, to compensate for the loss by evaporation. The mixture is allowed sufficient time for the precipitate to settle (about an hour). Now add an equal volume of 0.5 per cent. aqueous solution of yellowish eosin (filtered) to the methylene blue solution in a large evaporating dish.

[1] Wilson, T. M. "On the chemistry and staining properties of certain derivatives of the methylene blue group when combined with eosin." *Jour. Exp. Med.*, 1907, IX, 645.

[2] The cheaper grades of methylene blue may be used with satisfactory result.

[3] The silver oxid may be prepared by dissolving 2.0 gm. of silver nitrate in 15 c. c. of distilled water and adding to it 260 c. c. of calcium hydrate. Shake well, and set aside for the precipitate to settle. Decant the supernatant fluid, collect the precipitate on a filter, and wash with 20 to 25 c. c. of distilled water. Dry the precipitate at a temperature not exceeding 100° C., and place it in a brown bottle, tightly stoppered.

Mix the solutions well, and allow the mixture to stand one hour. Filter thrice, using a hard filter paper, such as the Schleicher and Schüll filter, No. 575, and finally wash the precipitate which has collected on the filter paper with physiological salt solution. (The precipitate which adheres to the evaporating dish is discarded.) Dry the precipitate in the thermostat, and transfer it to a dark bottle, tightly stoppered. The staining solution is then prepared by dissolving 0.4 gm. of the powdered precipitate in 100 c. c. of absolute methyl alcohol (Kahlbaum's). The stain may be rubbed in a mortar with the alcohol to facilitate solution, or powder and alcohol are placed in a bottle, which is vigorously shaken a few minutes on several successive days. The staining solution should be preserved in a dark bottle with glass stopper. (Wilson advises the use of 0.3 gm. of the dry stain to 100 c. c. of denatured alcohol, but in our hands this has not given satisfaction.) It is best to make up small quantities of the stain at frequent intervals (3 to 4 months).

Method of Staining.—As the methyl alcohol in which the stain is dissolved is apt to run over the edge of the cover glass, it is advisable to use the usual wire staining forceps; when staining on glass slides, the stain may be confined to the area of the smear by drawing lines on the glass with a *blue* wax pencil—the red wax is usually loosened by the alcohol.

(a) Cover the *unfixed* blood film with 5 to 6 drops of the stain for 1 minute. As the stain is dissolved in absolute methyl alcohol, the blood is fixed by this procedure. Precipitation of the stain through evaporation of the alcohol will be troublesome, if too little stain is used.

(b) Add to the stain an equal number of drops of *distilled* water, and allow it to remain on the film 2 to 4 min-

utes. A metallic scum forms on the surface. (The exact proportion of stain and water should be determined for each new lot of stain. At times twice the quantity of water is necessary; occasionally, however, fewer drops of water than of stain are required, especially with old mixtures, which have become slightly acid.)

(c) Wash with *distilled* water, blot dry, and mount in neutral balsam. The specimen should be held level during the washing and the stream of water directed against the surface of the cover glass, so that the metallic scum and precipitate in the fluid will be *floated* off. *Avoid dumping the stain from the cover glass,* for the precipitate adheres to the corpuscles, and cannot be removed by washing in water. If there is precipitate in the specimen, it may be removed by immersing the preparation momentarily in absolute methyl alcohol or ethyl alcohol, but always at the risk of decolorizing the cells too much; it is particularly the basic stains, methylene blue and methylene azure, which are decolorized.

A properly stained blood film should have a pinkish-gray or gray color when dry. If the color of the film is bright pink, the specimen is usually not satisfactory, or, rather, it is capable of being improved upon. The *erythrocytes* are stained very pale pink (plate II) or mauve or grayish-pink. Polychromatophilia is denoted by varying admixtures of blue. In extreme grade a polychromatophilic red cell is dark blue, no trace of pink being discernible. Basophilic granules stain dark blue. Occasionally, particularly in the blood of pernicious anemia, fine granules are seen which are stained violet or purple. Nuclei are stained purple. Nuclear particles stain like the nuclei, while the ring bodies are usually violet or reddish-purple. The differentiation of these abnormalities is more striking

LEGEND FOR PLATE II.

(All drawings made with camera lucida; × 1200. Wilson's stain, modified Romanowsky.)

1. Normal red corpuscle.
2. Pale or anemic corpuscle.
3. Basophilic granules in erythrocyte.
4. Nuclear particle (Howell's body) in erythrocyte.
5. Erythrocyte containing nuclear particle and basophilic granules.
6. Polychromatophilic red cell containing a Cabot's ring body.
7. Normoblast.
8. Slightly polychromatophilic erythrocyte containing a nuclear particle, Cabot's ring body, and violet colored basophilic granules.
9. Normoblast showing an early stage of karyorrhexis.
10. Megaloblast, markedly polychromatophilic.
11. Poikilocyte, markedly polychromatophilic and exhibiting reddish basophilic granules.
12. Blood platelets.

13, 14. Small lymphocytes.

15. Large lymphocyte, exhibiting azurophilic granules.
16. Large mononuclear leukocyte with a few azurophilic granules in the cytoplasm.
17. "Transitional" leukocyte with fine azurophilic granules.
18. Polynuclear neutrophilic leukocyte.
19. Polynuclear eosinophilic leukocyte.
20. Mast cell or polynuclear basophilic leukocyte.
21. Neutrophilic myelocyte.

PLATE II

and brilliant with the Romanowsky stains than with any of the other blood stains. Cabot's ring bodies are usually demonstrable only with Romanowsky stains, i. e., they are stained with methylene azure.

Blood platelets are well brought out with Romanowsky stains alone. The granular chromatin masses are stained reddish-purple, the body of the platelet being unstained or exhibiting varying shades of blue.

All *leukocytic nuclei* are beautifully stained, the color being a deep reddish-purple. The morphology of the nucleus is well demonstrated. The leukocytic *granules,* on the other hand, stain poorly and very uncertainly, so that differential counting may be difficult or well-nigh impossible when pathological cells are present. The neutrophilic granules are stained lilac, but often only a few of the granules—sometimes none of them—take the stain. Eosinophilic granules are pink, but stain very uncertainly; at times there may be considerable doubt as to their nature. Basophilic granules are colored purple. The granules of the myelocytes usually are purple, regardless of the variety of the cell, though eosinophilic myelocytes may show a shade of pink in the granules. The cytoplasm of lymphocytes is colorless or blue—at times a very dark blue. In about one-third of the lymphocytes of normal blood purplish granules, varying much in size and number, are evident in the cytoplasm. These granules are demonstrable only with methylene azure, and are, therefore, designated *azurophile* granules. The large mononuclears and transitionals are, like the lymphocytes, more beautifully demonstrated with Romanowsky stains than by any other means. The cytoplasm of the large mononuclears is colorless or blue and generally non-granular, though it is now and then seen to be filled with azurophilic granules, which are for the most part very

fine and dust-like. In the case of the transitional leukocytes the protoplasm is studded with very fine azurophilic granules practically without exception. When these granules are observed in a large mononuclear cell, the resemblance it bears to a myelocyte is close at first glance. It is seen, however, that, while similar in color, the myelocytic granules are rather coarser, and close inspection will usually reveal the granules over the nucleus in the myelocyte, a point which serves to differentiate them from the large mononuclears. Usually, too, the relations between nucleus and protoplasm are different in the two types of cell. (With the triacid stain and with Jenner's stain this difficulty never arises, since the large mononuclears are non-granular.)

Besides precipitated stain in the specimen, which may be avoided as indicated above, the chief *difficulty in the application of the Romanowsky stains* arises in understaining with the basic components of the mixture. When this occurs, the erythrocytes are bright pink or red, the nuclei of the leukocytes blue instead of purple, and the chromatin of the platelets blue or unstained, while the chromatin of malarial or other parasites is entirely unstained. Such a condition may be due to one or more causes: (1) Dilution of the stain with too much water interferes with the nuclear staining. The requisite proportions of stain and water must be learned by experiment. If the nuclei are poorly stained, try less water. (2) Even a trace of acid in the water used for diluting or washing will weaken or remove the basic stains more or less completely. Staining in a room in which there are acid fumes is at times sufficient to ruin the specimen. (3) Acidity of the staining mixture itself may explain the failure of the nuclear stain. A minute quantity of acid in the bottle (or on the cork) in

which the stain is placed, or the formation of formic acid from the methyl alcohol, are possible sources of difficulty. Staining mixtures which contain acid may be made perfect, according to Peebles and Harlow,[1] by the addition of a few drops of absolute methyl alcohol in which a small quantity of potassium hydrate (by alcohol) has been dissolved. In case too much alcohol has been added, as shown by overstaining with the blue, it may be cautiously titrated back with absolute methyl alcohol containing a trace of glacial acetic acid. (4) Prolonged washing may spoil the result. It can readily be demonstrated by experiment that washing in water tends to remove the basic (blue) elements of the stain, thus rendering the eosin more conspicuous. With a good staining mixture and the right proportion of stain and water, it is only necessary to wash long enough to remove the excess of stain; the specimen is then blotted dry to prevent further decolorization.

Overstaining with the blue is seldom a source of difficulty, except in old smears. When it does occur, it may be corrected by one of the four procedures just described. With very old blood films, it may be impossible to prevent diffuse blue staining of the red corpuscles, though Brem's method (p. 175) or Giemsa's slow method may give good results.

(b) Leishman's Stain.—This stain is most conveniently obtained in tablet form from Burroughs, Wellcome, & Co. or from Grübler. Grübler also supplies the powdered stain in bulk. A tablet is dissolved in a stated quantity of absolute methyl alcohol, and the mixture is then ready for use. It should be kept in a tightly stoppered bottle.

The staining technique is practically identical with that

[1] Peebles, A. R., and Harlow, W. P. "Clinical observations of blood stains." *Jour. A. M. A.*, 1909, LII, 768.

given for Wilson's stain. The nuclei have a little more of a reddish hue, but otherwise the picture is much the same. The advantages and limitations of the stain are those given above.

(c) GIEMSA'S STAIN.—The preparation of Giemsa's stain is difficult. Grübler & Co. supply a reliable solution. The staining mixture contains eosin, azure I, and azure II.

Method of Staining.—(a) Fix the specimen in absolute methyl or ethyl alcohol.

(b) Add one drop of the staining mixture to 1 c. c. of distilled water. (This must be freshly prepared.) Stain 10 to 30 minutes with this dilution of the staining mixture.

(c) Wash with distilled water, blot dry, and mount in balsam.

The appearance of the stained film is the usual Romanowsky picture (see p. 286). The nuclei, however, are a little redder than usual, and the neutrophilic granules are even less uniformly stained than with most other Romanowsky stains.

In case the chromatin staining is unsatisfactory, a very dilute solution of sodium carbonate may be substituted for distilled water in preparing the dilution of the stain.

OLD BLOOD FILMS.—Blood films which have been kept for several months before staining them do not give good results with the usual Romanowsky procedures. The difficulty lies chiefly in the staining of the erythrocytes, which take a diffuse, slate-blue color. To avoid this to a great extent, the films may be stained with Leishman's or Wilson's stain, using Brem's technique (p. 175), or with Giemsa's stain. With the latter the dilution of the stain should be one drop to five or more cubic centimeters of

water. The specimens are allowed to remain in this fluid 24 to 48 hours. They are then washed in water and mounted as usual. With this procedure it is often possible to demonstrate the chromatin of malarial parasites in specimens a year or more old.

(7) **Jenner's Stain**[1] (the eosinate of methylene blue).—If one stain alone were to be selected for the general routine examination of the blood, Jenner's would probably be the choice of most workers. It is a much better stain for nuclei and for pathologic alterations in the red corpuscles than Ehrlich's triacid, though inferior to the Romanowsky stains in these respects. On the other hand, it is much superior to the Romanowsky stains for the demonstration of the granules in leukocytes, though surpassed for this purpose by Ehrlich's triacid mixture.

Preparation of the Stain.—The tablets of Jenner's stain, which have been placed upon the market by Burroughs, Wellcome, & Co. and by Grübler, have done away with the necessity of making the stain, and have made it possible to have on hand fresh solutions of the staining mixture. The tablets are dissolved in a stated quantity of methyl alcohol; the solution is ready for use at once. Numerous modifications of Jenner's methods of preparing the stain have been described, without, however, simplification or improvement of the original procedures. Jenner's methods are as follows:

(a) *First Method.*—Prepare a 1.2 per cent. to 1.25 per cent. solution of Grübler's water-soluble, yellowish eosin in distilled water, and also a 1 per cent. aqueous solution of Grübler's medicinal methylene blue. Mix equal parts of the two solutions in an evaporating dish and, after stirring

[1] Jenner, L. "A new preparation for rapidly fixing and staining blood." *Lancet*, 1899, I, 370.

thoroughly, allow the mixture to stand for 24 hours. Filter through a hard filter paper (Schleicher and Schüll's No. 575), and dry the precipitate, which collects on the paper, either at room temperature or in the incubator at 37° C. The temperature may be as high as 55° C. without injuring the precipitate. The dried precipitate is removed from the filter paper, powdered in a mortar, shaken with distilled water, and the precipitate again collected on a filter paper. The washings should have a dirty purplish color. Finally, the precipitate is again dried and stored in brown glass bottles. For use dissolve 0.5 gm. of the powdered precipitate in 100 c. c. of absolute methyl alcohol, filter, and preserve in a tightly stoppered bottle. The solution keeps well.

(b) *Second Method.*—Instead of using aqueous solutions, Jenner found that the eosin and methylene blue may be dissolved directly in absolute methyl alcohol. The staining mixture is prepared by adding 125 c. c. of a 0.5 per cent. solution of Grübler's yellowish eosin in absolute methyl alcohol to 100 c. c. of a 0.5 per cent. alcoholic solution of Grübler's medicinal methylene blue. The mixture is ready for use immediately.

METHODS OF STAINING.—The following is the technique originally recommended by Jenner:

(1) Cover the *unfixed* blood film with the stain 1 to 3 minutes. To prevent evaporation and precipitation of the stain, the specimen is covered with a watch glass.

(b) Wash quickly in distilled water, blot dry, and mount in balsam.

A *second method of staining,* which often gives good differentiation of the granules of the leukocytes and polychromatic nuclear staining, is as follows:

(a) Cover the unfixed specimen with about 8 drops of stain for 2 to 3 minutes.

(b) Add to the stain about 10 drops of distilled water, and allow the mixture to remain on the preparation 1 to 2 minutes or longer.

(c) Wash with distilled water (observing the precautions given on page 286), blot dry, and mount in balsam.

With Jenner's stain the *red blood corpuscles* are terra cotta or pink. Polychromasia causes the cell to assume a bluish tint. Basophilic granules in the red cells are dark blue, nuclei and nuclear particles are of the same color, though usually somewhat more deeply stained. Cabot's ring bodies are unstained or pale blue.

The *blood platelets* are poorly stained. They are mauve in color and indistinct morphologically. With the second method, however, they are well differentiated, as with Romanowsky stains.

Leukocytic nuclei are stained dark blue (purple with the second method). The neutrophilic *granules* are red, often with a violet tint. Eosinophilic granules are also red, but they are distinguishable from the former by their greater size and brilliance of staining. Mast-cell granules are purple. The granular differentiation is less distinct in myelocytes than it is in polynuclear cells, as a rule, and is inferior to that obtained with Ehrlich's triacid stain.

The protoplasm of malarial parasites is stained light blue; chromatin is usually unstained.

[1] In staining specimens on glass slides, where the area of the film is larger, more stain should be employed to prevent too rapid concentration of the stain through evaporation. The relative proportion of stain to water should be preserved.

TABLE OF STAINING REACTIONS WITH EHRLICH'S TRIACID, ROMANOWSKY'S AND JENNER'S STAINS

Leukocytes.	*Ehrlich's triacid stain.*	*Romanowsky stains.*	*Jenner's stain.*
(a) Lymphocytes.	Nucleus pale green, cytoplasm colorless or pinkish.	Nucleus reddish purple. Cytoplasm blue or colorless. Azurophilic granules in cytoplasm of *ca.* 1/3 of lymphocytes.	Nucleus dark blue. Cytoplasm paler blue, at times darker than nucleus.
(b) Large mononuclears.	Nucleus *very* faintly green. Cytoplasm unstained or pale pink.	Nucleus reddish purple. Cytoplasm blue or colorless. Fine azurophilic granules in cytoplasm, at times.	Nucleus blue. Cytoplasm paler blue.
(c) Transitionals.	Same as (b).	Same as (b). Granules constantly present in cytoplasm.	Same as (b).
(d) Polynuclear neutrophiles.	Nucleus darker green or blue. Granules lilac.	Nucleus reddish purple. Granules lilac; often poorly stained or unstained.	Nucleus dark blue. Granules pink.
(e) Eosinophiles.	Nucleus same as (d). Granules copper colored.	Nucleus same as (d). Granules pink, often poorly differentiated.	Nucleus same as (d). Granules red.
(f) Mast cells.	Nucleus as in (d). Granules unstained, appear as vacuoles.	Nucleus same as (d). Granules reddish purple.	Nucleus same as (d). Granules purple.
(g) Myelocytes.	Neutrophile, eosinophile and basophile granules well differentiated.	Usually all granules stain reddish purple.	Granules fairly weil differentiated.
Platelets.	Poorly stained.	Well stained. Fine, purplish chromatin.	Poorly stained.
Red cells.			
(a) Polychromasia.	Not demonstrable.	Varying shades of blue.	Varying shades of blue.
(b) Basophilic granules.	Not stained.	Dark blue.	Dark blue.
(c) Nuclear particles.	Pale green or blue.	Reddish purple.	Dark blue.
(d) Ring bodies.	Not stained.	Violet or reddish purple.	Not stained or pale blue.
Malarial parasites.	Poorly stained. Chromatin unstained.	Chromatin red. Protoplasm light blue.	Chromatin usually unstained. Protoplasm light blue.

Methyl Green-Pyronin Mixture of Pappenheim.[1]—Saturated aqueous solutions of methyl green and of pyronin are made. One part of the pyronin solution is added to three to four parts of methyl green. When sufficient pyronin has been added, the mixture begins to take on a bluish tint. It is often possible to obtain very good staining mixtures from Grübler.

Method of Staining.—(a) Fix the blood films with heat.

(b) Stain 5 to 10 minutes.

(c) Wash quickly in distilled water, blot dry, and mount in balsam.

All nuclei are stained dark green or blue. Erythrocytes take a gray or slate color. Polychromatophilic cells are stained more or less intensely by the pyronin, and exhibit varying shades of red. Basophilic granules are stained a brilliant red, in marked contrast to the nuclei. Nuclear particles are stained dark green or blue,[2] like normoblastic nuclei, or red, like the basophilic granules.

The protoplasm of lymphocytes is stained red. Leukocytic granules are not specifically stained.

The Iodin Reaction of the Leukocytes.—This reaction, discovered by Ehrlich, is demonstrated as follows: (1) The air-dried blood films are placed in a mixture composed of:

Iodin	1.0 gm.
Potassium iodid	3.0 gm.
Distilled water	100.0 c.c.
Gum arabic, q. s. (to give a syrupy consistence).	

[1] Pappenheim, A. "Vergleichende Untersuchungen ueber die elementare Zusammensetzung des rothen Knochenmarkes einiger Säugethiere." *Virchow's Archiv*, 1899, CLVII, 19.

[2] Morris, R. S. "Nuclear particles in the erythrocytes." *Arch. Int. Med.*, 1909, III, 93. (The observations reported here were made on old films. Subsequent study of fresh material shows that nuclear particles, while not infrequently stained blue, are, nevertheless, often red.)

(2) The air-dried specimen, instead of being placed in the mixture given above, may be put in a small vessel, in which a few crystals of iodin have been placed. The reaction appears in a few minutes. The specimen is mounted and examined in syrup made of levulose.

Permanent specimens cannot be made by either procedure.

The erythrocytes are stained diffusely brown.

A positive reaction consists in brown staining, of varying degree, of the protoplasm of the polynuclear neutrophiles. Occasionally the lymphocytes and mast cells are stained, rarely the large mononuclears and eosinophiles, while myelocytes never give the reaction (Zollikofer).

Differential Counting of the Leukocytes

For a differential count of the leukocytes the first essential is a well-spread and stained blood film. Ehrlich's triacid or Jenner's *stain* should be employed in the majority of instances. When myelocytes are present in the blood Ehrlich's triacid should be used, though for the usual run of cases Jenner's stain gives satisfactory differentiation. The Romanowsky stains are unsatisfactory for differential counting, for the granular leukocytes are often poorly differentiated. The non-granular cells are, however, beautifully stained.

For special purposes the triacid or Jenner's stain may be inferior to others. In studying *eosinophilia*, when it is desired simply to follow the percentage of eosinophiles from day to day, hematoxylin or methylene blue combined with eosin may be used, always at the risk, however, of missing certain abnormal cells, if present. In *lymphoid leukemia*, where the lymphocytes may constitute 90 per

cent. or more of the leukocytes, it is advantageous to use a better nuclear stain than the triacid, and one which will differentiate between nucleus and protoplasm more effectively than Jenner's. The Romanowsky stains are the best for this purpose, for the staining is sharp and clear. Hematoxylin also gives excellent results, though the picture is less comprehensive, for all granules are unstained. The *large mononuclears* and *transitionals* are most satisfactorily demonstrated with the Romanowsky stains. The differentiation between nucleus and protoplasm is clear, the morphology of the nucleus is shown in great detail, and the azurophile granules are made evident.

For the differential count a *mechanical stage* is almost a necessity. The stained specimen is placed on the stage and examined with the $\frac{1}{12}$ -in. oil immersion objective and an eye-piece (such as Leitz No. 3 or No. 4), which gives a high magnification. In making the count it is, of course, essential that the cells be counted only once. This is accomplished through the use of the mechanical stage, the specimen being moved up and down, with a lateral shifting of the field at the end of each "row." At least 500 cells should be counted.

Normal Leukocytes.—The leukocytes of the blood may be classified as follows:

(1) *Lymphocytes* (pl. I, 5; pl. II, 13, 14, 15) are cells having a single round or oval nucleus—rarely a kidney-shaped nucleus—and a rather scanty rim of protoplasm. The protoplasm is non-granular, though about 30 per cent. of the lymphocytes of normal blood possess azurophile granules, which are demonstrable after staining with methylene azure, but not with other stains. These granules vary greatly in number and size. Often there are many granules, more frequently only a few, in a cell. The diam-

eter of lymphocytes varies between 7 and 11 micra. They constitute 22 to 25 per cent. of the leukocytes normally.

(2) *Large mononuclear leukocytes* (pl. I, 6; pl. II, 16) resemble the lymphocytes, but are larger and have relatively more protoplasm. The nucleus is somewhat poorer in chromatin and, therefore, stains less intensely. The protoplasm may contain azurophile granules, which are either very fine, like those of the transitionals, or rarely coarser, like the granules of the lymphocytes. The cells are actively ameboid and phagocytic—the *macrophages* of the blood. The diameter is 12 to 20 micra.

(3) *"Transitional" leukocytes* (pl. I, 7; pl. II, 17) differ from the large mononuclears in the shape of the nucleus, which is horseshoe-shaped, lobulated, or deeply indented, and in the constant presence in the protoplasm of numerous fine, dust-like azurophile granules. With stains other than the Romanowsky, both cells present non-granular cytoplasm, though exceptionally a few fine, faintly stained granules are discernible after staining with Ehrlich's triacid stain. These granules, when evident, are usually stained a reddish or pinkish tint.

The large mononuclears and transitionals together form about 3 to 5 per cent. of the leukocytes of normal blood. The diameter is the same as that of the large mononuclears.

(4) *Polynuclear neutrophilic leukocytes* (pl. I, 8; pl. II, 18) are cells with polymorphous nuclei, in whose cytoplasm are numerous fine, neutrophilic granules. These cells are about 9 to 12 micra in diameter and constitute 65 to 70 per cent. of the white cells under normal conditions. The cells are actively ameboid and phagocytic in the fresh blood and are designated *microphages,* in distinction to the macrophages or large mononuclears.

(5) *Polynuclear eosinophilic cells* (pl. I, 9; pl. II, 19)

are similar to the last group (4), except for the presence of coarse eosinophilic or acidophilic granules in the protoplasm. They resemble the neutrophiles in size and in the possession of ameboid activity. Normal blood contains about 2 to 4 per cent. of these cells.

(6) *Mast cells* (pl. I, 10; pl. II, 20) are polynuclear basophilic leukocytes. The nucleus is usually simply indented or lobulated. The protoplasm contains basophilic granules, which are somewhat variable in size, the majority being about as coarse as the eosinophilic granules. The cells measure about 10 micra in diameter. Normally about 0.5 per cent. of mast cells are found in the blood.

Pathological Leukocytes.—In addition to the foregoing cells, which go to make up the leukocytes of normal blood, there appear in disease immature cells, the precursors of the ripe leukocytes of normal blood.

(7) *Neutrophilic myelocytes* (pl. I, 11; pl. II, 21) are the antecedents of the polynuclear neutrophilic cells. They differ from the latter in having a round or oval or slightly indented nucleus. The protoplasm contains neutrophilic granules, which are often finer than those of the polynuclear cells. In the older myelocytes the granules are abundant, while very young cells may contain only a few. The nucleus is poorer in chromatin than that of the mature polynuclear cell. The cells are subject to great variation in size. The majority lie between 12 and 20 micra in diameter, though larger and smaller cells are encountered now and then. In the nucleus one to four nucleoli may be visible, particularly after staining with methyl green-pyronin or with Romanowsky stains. The nucleus is usually eccentrically situated.

(8) *Eosinophilic myelocytes* (pl. I, 12) resemble neutrophilic myelocytes in every respect aside from the dif-

ference in the granules. Frequently the immature eosinophilic granules exhibit basophilic tendencies, in that they are stained with basic dyes. Thus, in a Romanowsky preparation, some or all of the granules may take a dark blue or purplish tint.

(9) "*Mast*" *myelocytes* (pl. I, 13) are generally small and present basophilic granules in the protoplasm.

(10) *Metamyelocyte* is a term used to designate cells whose nuclei have passed beyond the kidney shape and already present more or less deep indentations. They are transition stages between the myelocyte and the polynuclear neutrophilic cells. They are not to be confused with the so-called transitional cells (which are, in reality, misbranded, as they represent transition forms to no type of cell, so far as is known, though it was originally supposed that they developed into the polynuclear neutrophiles, hence the name "transitional"). They are differentiated from the transitionals by the abundance of neutrophilic granules in their cytoplasm, when stained with Ehrlich's triacid or Jenner's stain. With Romanowsky stains, on the other hand, the granules of the metamyelocyte and transitional may be identical in color, but those of the transitional are much finer.

(11) *Promyelocytes* represent the earliest form of myelocyte, the cell with very few granules in its cytoplasm. The term is superfluous.

(12) *Myeloblasts* (non-granular marrow cells, undifferentiated cells of the marrow, lymphoid cells of the marrow, etc.) (pl. I, 14) are the parent cells of the myelocytes. They differ from the latter in the complete lack of cytoplasmic granules. The nucleus is similar to that of the myelocyte. The protoplasm is basophilic. The cells vary from the size of a lymphocyte to cells 20 micra in diameter.

(13) *Irritation forms* (Türk) are cells with round or oval nucleus, like that of the myelocyte, and rather abundant protoplasm, which is markedly basophilic and generally vacuolated.

(14) *Pathological lymphocytes.* In disease lymphocytes may depart considerably from the normal. The size is subject to much greater variation; the protoplasm is often greatly reduced in amount and at times is not demonstrable. The nucleus may be convoluted or indented, the so-called *Rieder cells.*

(15) *Megakaryocytes,* the giant cells of the bone marrow, are very rare in the blood. The nucleus is greatly convoluted, and the cytoplasm, with Romanowsky stains, exhibits fine, dust-like granules. The cells are very large in the bone marrow, but only the smaller examples pass the capillaries and appear in the circulating blood.

The Normal and Pathological Red Blood Corpuscles

Non-nucleated red cells are designated *erythrocytes,* the nucleated forms *erythroblasts.*

Erythrocytes.—(1)*Normocytes* (pl. I, 1, 2; pl. II, 1) are normal red blood corpuscles. In the fresh specimen they present the familiar form of biconcave discs, the center being paler, owing to the thinner layer of hemoglobin at this point. The normal cells stain with acid dyes (orthochromatic). The average diameter is 7.5 micra.

(2) *Microcytes* are abnormally small erythrocytes.

(3) *Macrocytes* or *megalocytes* are abnormally large erythrocytes. They may be pale, swollen corpuscles or abnormally rich in hemoglobin. The diameter may amount to 25 micra. Abnormal variation in size of the corpuscles

is designated *anisocytosis.* Ameboid movements may be observed in some of the cells.[1]

(4) *Poikilocytes* (pl. II, 11) are cells of irregular form. The shape may be practically anything.

Erythroblasts.—*Nucleated forms* of the red blood corpuscle may be subdivided as follows:

(5) *Normoblasts* (pl. I, 4; pl. II, 7, 7, 9) are nucleated red corpuscles, having the diameter of the average normocyte. The nucleus is round, at times eccentric, and usually very dense (pyknotic) and rich in chromatin. Younger forms present nuclei with a visible chromatin network.

(6) *Microblasts* are abnormally small, nucleated red cells.

(7) *Megaloblasts* (pl. I, 3; pl. II, 10) are large erythroblasts, possessing a large oval or round nucleus. The diameter of the nucleus exceeds that of a normal red cell (Emerson). In the youngest forms the nucleus exhibits a beautiful chromatin network, with markedly basophilic protoplasm. More mature megaloblasts are more nearly orthochromatic and the nucleus is more homogeneous, the network being less conspicuous or lacking. Mitoses may be observed.[2] Ameboid activity may be seen in fresh specimens.[3]

(8) *Intermediates* is a term used to designate all those nucleated red corpuscles which can be classified neither as normoblasts, megaloblasts, nor microblasts (Emerson).

Abnormalities in the Staining of the Red Corpuscles.—(1) *Polychromasia* or *polychromatophilia* (pl. II, 6, 8, 10,

[1] Morris, R. S., and Thayer, W. S. "Amœboid movements in macrocytes and megaloblasts." *Arch. Int. Med.*, 1911, VIII, 581.

[2] Dock, G. "Mitosis in circulating blood." *Trans. Assoc. Amer. Phys.*, 1902.

[3] Thayer, W. S. "The amœboid activity of megaloblasts." *Arch. Int. Med.*, 1911, VII, 223. *See also* Morris, R. S. and Thayer, W. S. *Loc. cit.*

11) is a condition in which the red corpuscles stain with basic dyes. Normally the red cells possess an affinity for acid dyes, such as eosin. Polychromasia varies greatly in degree. With slight grades the color of the acid dye still predominates, though the tint of the basic dye is visible. When polychromasia is marked there is an intense staining of the cell with the basic dye alone. It occurs in both erythrocytes and erythroblasts.

(2) *Basophilic granulation* or *stippling* (pl. II, 3, 5) is seen in non-nucleated red cells and in nucleated corpuscles, often with intact nuclei. As the name implies, the cell contains granules which are stained only with basic dyes. The granules are always quite numerous in the cell and vary considerably in size. As a rule, the smaller the granules the more numerous they are. With the variation in size there is also irregularity of form. Generally the larger granules are observed in orthochromatic cells, the smaller in polychromatophilic cells, as Askanazy has pointed out.

(3) *Fragmentation of the Nucleus.*—Karyorrhexis (pl. II, 9), the breaking up of the nucleus within the cell, leads to characteristic appearances. The fragmented nucleus may resemble a rosette, or the nucleus may resolve itself into a number of round or oval, often irregular, masses, which are united or separate from one another.

(4) *Nuclear Particles.*—Nuclear particles (pl. II, 4, 5, 8) are derived from the nucleus of the red cell through atrophy of the nucleus or by karyorrhexis; it is possible that there is another mode of formation, since nuclear particles may be found in megaloblasts with active, intact nuclei. They were first observed in the blood of the cat by Howell,[1] and are known as Howell's bodies or Howell's

[1] Howell, W. H. "The life-history of the formed elements of the blood, especially the red corpuscles." *Jour. Morphol.*, 1890, IV, 57.

nuclear particles. They occur also in human blood.[1] They are small, round, sharply defined bodies, usually situated eccentrically in the cell, and generally occur singly, though as many as nine have been observed in a non-nucleated red corpuscle. They resemble miniature pyknotic nuclei morphologically.

(5) *Ring Bodies.*—Ring bodies (pl. II, 6, 8) were first described by Cabot,[2] and are usually designated Cabot's ring bodies. The ring may remain round or may be twisted so as to form a figure eight, etc. The rings are best seen after staining with Romanowsky stains, which usually color them red or reddish-violet, rarely blue. It is believed by Cabot, and by all who have since studied these bodies, that they represent the nuclear membrane.

(6) *"Red Basophilic Granulation with Romanowsky Stains"* (pl. II, 8, 11).—Naegeli[3] in particular has called attention to the existence of a granulation seen in specimens stained with Giemsa's stain; it is demonstrable with all Romanowsky stains. The granules differ from the usual basophilic granules, in that they are stained red or violet instead of blue. The granules were observed by Cabot in cells which also contained ring bodies. Naegeli believes that the granules originate from the nucleus, probably from the nuclear membrane, since ring bodies may be made of a series of dots similarly stained.

(7) *Schüffner's Granules.*—Schüffner's granules are

[1] Morris, R. S. (a) "Note on the occurrence of Howell's nuclear particles in experimental anemia of the rabbit and in human blood." *Johns Hopkins Hosp. Bull.*, 1907, XVIII, 198. (b) "Nuclear particles in the erythrocytes." *Arch. Int. Med.*, 1909, III, 93.

[2] Cabot, R. C. "Ring bodies (nuclear remnants?) in anemic blood." *Jour. Med. Research*, 1903, IX, 15.

[3] Naegeli, O. "Blutkrankheiten und Blutdiagnostik." Leipzig, 1912, 2nd Ed., p. 153.

found only in certain cases of malaria. They are seen in the infected corpuscles (see below).

Demonstration of Protozoa in the Blood

The blood protozoa which are of pathological importance are few in number. At the present time the plasmodia of malaria alone demand general consideration in this country. For protozoa in general, however, such as trypanosomes, Leishman-Donovan bodies, etc., the method of demonstration in the stained specimen, which is universally employed, is one of the numerous modifications of the Romanowsky stain (q. v.).

Malarial Parasites.—The *plasmodia of malaria* are characteristically stained by the Romanowsky method. The *protoplasm* of the parasite is stained light blue, contrasting well with the pink color of the red corpuscle. The *nuclear chromatin* of the parasite is colored a brilliant red or purplish-red, while the *pigment* retains its original color, being unstained.

In *certain* of the infections with Plasmodium vivax and Plasmodium falciparum, peculiar granulations appear in the infected red corpuscles (Schüffner's granules). They have been described by Schüffner and others. With Romanowsky stains, the granules exhibit a dark, reddish tint, often quite like that of the chromatin of the parasite. *Schüffner's granules* are not to be confused with the ordinary basophilic granules of the red cells or with the red granulations seen in certain corpuscles when stained with Romanowsky stains. By means of vital staining Boggs [1] has adduced further proof of the non-identity of Schüff-

[1] Boggs, T. R. "Vital staining of 'stipple cells' in malarial blood." *Jour. A. M. A.*, 1911, LVII, 150.

ner's with other granules. The granules may be missed in cells containing the youngest hyalin parasites, and usually seem to increase in number with the age of the parasite. Schüffner's granules have not been observed in cells infected with the parasite of quartan fever.

Blood platelets have been mistaken for hyalin forms of the plasmodia by inexperienced observers. This is apt to occur only when the platelet rests upon the red corpuscle. Differentiation is simple. The chromatin of the platelet is usually colored purple with less of the reddish tint than the chromatin of the parasite shows, but this difference may be lacking, for often the chromatin of the parasite is stained exactly the shade of that of the platelet. The important differential point is found in the arrangement of the chromatin. In the platelet the chromatin is scattered in minute granules, while the chromatin of the *hyalin* parasite is in a compact mass, or, in the case of Plasmodium falciparum, in two or three masses, but still dense and compact. The body of the platelet is often unstained, but may take a pale blue color, very much like that of the protoplasm of the parasite. Giant blood platelets have been mistaken for extracellular forms of the malarial parasite. The constant presence of pigment granules in the parasite should be sufficient to differentiate, even though the distribution of the chromatin in this instance be somewhat similar in the two—which is generally not the case.

STAINING METHOD OF ROSS.[1]—Ross has devised a method for detecting the parasites which is useful when their number in the blood is small. He prepares a thick smear of the blood and, before staining the film, extracts the greater part of the hemoglobin from the cells. A drop of blood of

[1] Ross, R. "An improved method for the microscopical diagnosis of intermittent fever." *Lancet*, 1903, I, 86.

about 20 c. mm. is placed on a cover glass (¾ in. square) and spread in the usual manner, or with a needle or lancet. It is dried in the air. The preparation contains about twenty times the amount of blood usually found in a smear. After becoming dry it is covered with 1 per cent. eosin solution (Romanowsky's). The stain is placed on the specimen with a glass rod and is allowed to act for as much as fifteen minutes. It is then washed. The washing must be done with a very gentle stream, since the unfixed blood is easily loosened. Then stain with Romanowsky's methylene blue [1] a few seconds and again wash carefully. The specimen is dried in the air and mounted in balsam. The hemoglobin is extracted from the red corpuscles, leaving only their stromata, together with leukocytes, platelets, and plasmodia. The staining of the parasite is distinctive—protoplasm light blue and nuclear chromatin red. Hyalins are readily detected.

For finding larger, pigmented parasites Ross prepares the film as described above; after it has dried, it is covered with distilled water to extract the hemoglobin. The unstained specimen is then examined. Crescents and other pigmented forms stand out prominently.

Ruge's Modification [2] of the Method of Ross.—A disadvantage in the method of Ross is the difficulty of washing the unfixed specimen without losing the preparation. To overcome this, Ruge fixes the thick films in 2 per cent. formalin containing ½ to 1 per cent. acetic acid. The hemoglobin is extracted from the erythrocytes, which are fixed to the cover glass at the same time. The specimen

[1] Nocht's solution of methylene blue for the Romanowsky stain consists of methylene blue (rectif. puriss. Hoechst) 1.0 gm., sodium carbonate 0.5 gm., dissolved in 100 c. c. of distilled water.

[2] Ruge, R. "Zur Erleichterung der mikroskopischen Malariadiagnose." *Deutsche med. Wchnschr.*, 1903, XXIX, 205.

is then stained with Romanowsky's stain. The formalin fixation interferes somewhat with the staining of the protoplasm of the plasmodia, so that it may be necessary to restain the film with methylene blue. A certain amount of precipitate remains in the specimen, but the parasites are well stained.

Examination of the fresh blood, whenever possible, is the most satisfactory method of diagnosis of malaria, as the variety of the parasite is more easily recognized, as a rule, than in stained smears. The accompanying table gives the main differences between the three species of plasmodia in fresh blood; it may be used also, with certain obvious exceptions, in connection with stained smears.

Degenerations in the red cells may be mistaken for hyalin parasites. In form the degenerations may bear a striking resemblance to ring forms or irregularly shaped parasites. Ameboid activity is lacking, and it may be noted that the degenerations become more numerous in the specimen as time advances. The larger round or oval degenerations are less confusing; their size appears to change on raising and lowering the focus.

Examination of the Blood for Animal Parasites

(1) **Filaria bancrofti** (Fig. 10), when present in the blood, is usually demonstrable by the ordinary method of examination of the fresh blood. The size of the drop should be a little larger than usual, in order to secure a moderately thick preparation. The blood should be taken during the sleeping hours—usually at night, when the embryos are

The Appearances of Malarial Plasmodia in Fresh Blood

	Pl. vivax (tertian).	*Pl. falciparum (estivoautumnal).*	*Pl. malariæ (quartan).*
I. Hyalins.			
(a) Shape.	Often irregular, occasionally ring forms.	Usually ring forms, occasionally irregular.	Irregular or ring forms.
(b) Refractivity.	Difficult to see; much like the red cell. Ring forms more refractive.	Easily seen; refractive.	Rather easily seen.
(c) Motility.	Actively ameboid.	Occasionally active. Rings sluggish.	Sluggish usually.
(d) Multiple infections.	Infrequent.	Frequent. May be six or more hyalins in a single cell.	Infrequent.
II. Pigmented forms. (a) Shape.	Ameboid, very irregular. Three-quarters and full grown parasites round.	Usually no pigmented forms in circulating blood. Round or oval, when seen.	Irregular, soon becoming round or oval. "Band" forms not infrequent.
(b) Refractivity.	Young forms difficult to see.	Easily seen; refractive.	Easily seen.
(c) Motility.	Young forms actively ameboid.	Sluggish.	Sluggish.
(d) Pigment.	Fine brown granules scattered throughout parasite.	Fine, dark brown granules, centrally placed.	Coarse brown granules, peripherally placed.
(e) Motility of pigment.	Very active in younger forms.	Sluggish.	Very sluggish.
(f) Merozöites or daughter parasites.	12 to 24, usually about 16.	8 to 24, usually 12 to 16, small.	6 to 12, often 8.
III. Sexual forms. Shape.	Round.	Usually crescentic, at times round or oval.	Round.
IV. Infected red cells.	Swollen and pale.	Often brassy, shrunken or crenated.	Often brassy, no swelling.

present in the blood vessels of the skin. The wriggling motion of the embryos arrests attention at once. Fair permanent specimens may be secured by making a relatively thick smear of the blood, which is then stained with hematoxylin and eosin, or one of the Romanowsky stains. Stäubli's method is useful when the parasites are scarce. The embryos are 0.125 to 0.300 mm. long and 0.007 to 0.011 mm. thick (Blanchard).

(2) **Trichinella Spiralis.**—The embryos of Trichinella spiralis are at times found in the blood. The method of detecting their presence is that which Stäubli [1] evolved in the study of experimental trichinosis, and which has been successfully applied to the diagnosis of human infections by Herrick and Janeway [2] and others. In human cases it is apparently desirable to employ larger quantities of blood than Stäubli found necessary.

Method of Stäubli.—By means of an aspirating syringe, 1 to 10 c. c. of blood are withdrawn from an arm vein with the usual aseptic technique. The blood is immediately laked by adding it to about 15 volumes of 3 per cent. acetic acid, shaking thoroughly to prevent clotting. The acid is strong enough to insure complete laking of the erythrocytes, and at the same time is not injurious to the embryos. The mixture is centrifugalized, the sediment removed with a pipette, and examined fresh. It consists mainly of leukocytes. The specimen should be examined with a mechanical stage, so that all parts of it may be inspected if necessary. Moderately high magnification should be employed. At the time of their entrance into the circulation the embryos are

[1] Stäubli, C. "Beitrag zum Nachweis von Parasiten im Blut." *München. med. Wchnschr.*, 1908, LV, 2601.

[2] Herrick, W. W., and Janeway, T. C. "Demonstration of the Trichinella spiralis in the circulating blood in man." *Arch. Int. Med.*, 1909, III, 263.

about 0.120 to 0.160 mm. long and 0.007 to 0.008 mm. in thickness (Blanchard).

Stäubli has obtained good permanent specimens by staining the sediment with Giemsa's or Jenner's stain. The acetic acid should be completely removed by washing before attempting to use either of these stains.

CHAPTER VI

PUNCTURE FLUIDS

The methods described below are applicable to most puncture fluids, e. g., pleural, peritoneal, pericardial, hydrocele, etc. The examination of the cerebrospinal fluid, however, requires certain special procedures, which are considered separately.

Specific Gravity.—The specific gravity is usually determined only approximately with the urinometer. In filling the cylinder, special care is required to avoid bubbles on the surface because of the albuminous nature of the fluid. The determination should be made at once, before clotting of the fluid will have occurred. Because of the high temperature of the fluid immediately after withdrawal (approximately that of the body), a correction of the urinometer reading should be made. Most instruments are standardized for a temperature of 15° C. For each three (3) degrees C. above this temperature the specific gravity is depressed 0.001. The result is that the reading obtained on a perfectly fresh fluid is too low. (It must be remembered, however, that the values obtained with the clinical urinometer are, at best, only approximately correct.)

Albumin Content. Albumin content refers to the entire coagulable protein. For clinical use, the best method of determining the quantity of the coagulable protein is Tsuchiya's modification of the Esbach method (p. 35). It is necessary to dilute the fluid to such an extent that the reading in the Esbach tube will be 4 or less; in other

words, the dilution must reduce the albumin below 0.4 per cent. With higher percentages of protein the method is subject to considerable error. The diluted fluid is weakly acidified with acetic acid, and the determination is carried out in the same manner as with urine. The reading at the end of 24 hours is multiplied by the dilution, and the result is the number of grams of coagulable protein per 1,000 c. c. of fluid.

Tsuchiya's method is less accurate with puncture fluids than with urine.[1]

Accurate determinations of the coagulable protein may be made in connection with the estimation of the incoagulable nitrogen (see below). Five c. c. of the puncture fluid (before clotting has occurred) are measured by means of a pipette into each of two Kjeldahl flasks. To the fluid in each flask add about 15 c. c. of concentrated sulphuric acid, about 0.2 gm. of copper sulphate crystals, and about 10 gm. of potassium sulphate. The usual Kjeldahl determination of total nitrogen is now made (p. 20). The distillate is collected in 50 c. c. of $\frac{N}{10}$ sulphuric acid. The total nitrogen in grams per cent. is calculated. From this the incoagulable nitrogen in grams per cent. is subtracted. The difference between the two, multiplied by the factor 6.25, gives the coagulable protein in grams per cent.

The Incoagulable Nitrogen.—The accurate determination of the incoagulable nitrogen in puncture fluids is too time-consuming at present to be applied generally by clinicians. The method, an adaptation from Hohlweg and Meyer, is as follows:[2] To 10 c. c. of the puncture fluid in a 300-c. c. Erlenmeyer flask add a reagent composed of

[1] Mattice, A. F. Personal communication.

[2] Morris, R. S. "The incoagulable nitrogen of puncture fluids, with special reference to cancer. A preliminary note." *Arch. Int. Med.*, 1911, VIII, 457.

equal parts of 1 per cent. acetic acid and 5 per cent. solution of monocalcium phosphate, until the reaction is acid to litmus, but still neutral to Congo red. The limit is rather wide, varying from 2 to 6 or more c. c. with different fluids. The amount must be determined separately for each fluid. Distilled water is now added to bring the volume to 80 c. c., and then 80 c. c. of saturated aqueous solution of sodium chlorid are poured into the flask. The mixture is now boiled to precipitate the coagulable proteins, and is then filtered through a folded filter (Schleicher and Schüll's No. 589, blue ribbon) directly into a Kjeldahl flask. The Erlenmeyer flask and filter are washed three times with distilled water. A Kjeldahl nitrogen determination is made on the filtrate and washings. Owing to the quantity of sodium chlorid contained in the filtrate, a considerable excess of sulphuric acid must be added to convert the sodium chlorid into sodium sulphate and still leave sufficient sulphuric acid for the oxidation. For this purpose about 30 c. c. of concentrated acid are enough. Because of the preformed sodium sulphate, it is unnecessary to add potassium sulphate. After adding about 0.2 gm. of copper sulphate crystals, the oxidation and distillation are carried out as described under Kjeldahl nitrogen determination (p. 20). For collecting the distillate 15 c. c. of tenth normal sulphuric acid are used. All determinations are made in duplicate or triplicate, after a preliminary test of the filtrate with heat and dilute acetic acid has shown that the proteins are completely removed.

The nitrogen is calculated in grams per cent.

In the vast majority of fluids it will be found that the incoagulable nitrogen is below 0.0699 gm. per cent.—usually considerably below this figure. Values between 0.07 and 0.0899 gm. per cent. are of doubtful significance, but,

when the incoagulable nitrogen exceeds 0.09 gm. per ce the probability is strong that the fluid is either cancerous or sarcomatous in origin. It should be kept in mind, however, that lower values by no means exclude malignant disease.

A Protein Precipitable in the Cold by Dilute Acetic Acid.—Runeberg [1] was the first to apply to the diagnosis of puncture fluids Paijkull's observation on the presence of a protein precipitable by dilute acetic acid in the cold. He found that inflammatory exudates and those resulting from malignant neoplasms of the serous membranes usually contain this body in abundance, whereas transudates do not, or at most possess it only in traces. His observations have been confirmed by Umber,[2] Stähelin,[3] and others. Umber has designated the body in question "serosamucin."

METHOD.—About 10 c.c. of the fluid in a test tube are treated with a few drops of dilute acetic acid (3 per cent.) until the reaction becomes acid to litmus. A positive reaction is denoted by the appearance of a rather marked cloudiness. A slight cloud is of no significance, and is seen in transudates frequently. Previous clotting of the fluid apparently does not interfere with the test. A great excess of acetic acid may redissolve the precipitate, and is, therefore, to be avoided.

Cytology.—As is the case with the blood leukocytes, cells are occasionally seen in exudates and transudates which cannot be definitely classified, but, for the most part,

[1] Runeberg, J. W. "Von der diagnostischen Bedeutung des Eiweissgehaltes in pathologischen Trans- und Exsudaten." *Berlin. klin. Wchnschr.*, 1897, XXXIV, 710.

[2] Umber, F. (a) "Ueber autolytische Vorgänge in Exsudaten." *München. med. Wchnschr.*, 1902, XLIX, 1169. (b) "Zum Studium der Eiweisskörper in Exsudaten." *Ztschr. f. klin. Med.*, 1903, XLVIII, 364.

[3] Stähelin, R. "Ueber den durch Essigsäure fällbaren Eiweisskörper der Exsudate und des Urins." *München. med. Wchnschr.*, 1902, XLIX, 1413.

the cells of puncture fluids may be placed in the following groups:

(1) *Lymphocytes.*—Often cells similar to, and, doubtless, identical with, the small lymphocytes of the blood are numerous in puncture fluids. They are characterized by their small size, relatively large nucleus, surrounded by a narrow rim of protoplasm. Frequently the protoplasm is so scanty that the nucleus appears to be, and sometimes is, naked. All transitions in size from the lymphocyte to the endothelial cell may be observed.

(2) *Endothelial Cells.*—These are usually very large cells, with abundant protoplasm and one or more round or oval nuclei, rather poor in chromatin. Frequently several cells are seen *en masse.* The size of the cells, as well as their shape, is variable; smaller forms are not infrequent. The cytoplasm may exhibit degenerative changes.

(3) *Polynuclear neutrophile cells* are similar to those of the blood, which is their source. They are often well preserved, but degenerations are common both in protoplasm and nucleus. The polymorphous nucleus may come to resemble a single round nucleus.

(4) *Eosinophile cells* are much less commonly met with than any of the foregoing. They may, at times, form a striking feature of the cell picture.

(5) *Mast cells* are rare in puncture fluids.

(6) *Tumor cells,* as such, are not recognizable. The presence of many mitotic nuclei, however, as Dock[1] was among the first to point out, is highly suggestive. Frag-

[1] Dock, G. "Cancer of the stomach in early life and the value of cells in effusions in the diagnosis of cancer of the serous membrane." *Amer. Jour. Med. Sci.*, 1897, CXIII, 655. *Also* Warren, L. F. "The diagnostic value of mitotic figures in the cells of serous exudates." *Arch. Int. Med.*, 1911, VIII, 648.

ments of tissue, when obtained, should be hardened and studied in section.

(7) *Red blood corpuscles,* often well preserved, though frequently crenated or otherwise degenerated, are often found in puncture fluids, particularly when of tuberculous or malignant origin.

METHOD OF OBTAINING CELLS.—The fluid should be centrifugalized at high speed before clotting has occurred. The cells are then removed from the bottom of the centrifuge tube with a pipette, spread on glass slides, dried, fixed, and stained. One of the Romanowsky stains, Jenner's stain, or hematoxylin and eosin may be employed.

In case the fluid cannot be centrifugalized immediately after its withdrawal from the body, about 10 c. c. of it should be discharged into an equal volume of 1 per cent. sodium fluorid solution to prevent clotting. For the sodium fluorid there may be substituted a 1.5 per cent. solution of sodium citrate in 0.85 per cent. sodium chlorid. Examination of the cells should be made within a day.

Tubercle bacilli may be sought in puncture fluids, but generally without success. *Inoculation* of the fluid, obtained under aseptic precautions, into the peritoneal cavity of a guinea-pig or rabbit is the best method of determining the presence or absence of tubercle bacilli. About 10 c. c. of fluid are injected. It is important that the fluid be injected before clotting has begun. Young animals are somewhat more susceptible to infection. After an interval of two to six weeks, the animal is sacrificed, and autopsy is performed, to determine the presence or absence of tuberculous lesions.

CEREBROSPINAL FLUID

Lumbar puncture often yields only a few cubic centimeters of cerebrospinal fluid, though at times 50 c.c. or more may be safely withdrawn. The determination of specific gravity, which is normally between about 1.006 and 1.010, is of little importance clinically. The number and kind of cells, the globulin content, and the bacteriological findings are the chief points of interest.

Cells of the Cerebrospinal Fluid.—The cerebrospinal fluid normally contains very few cells. Indeed, some observers have reported no cells in the normal fluid, though such a finding is probably exceptional. From 1 to 7 cells per cubic millimeter has been given as the normal limit.[1] More than 10 cells per c. mm. is pathological.[2] The cells are chiefly lymphocytes. In disease pus cells may be numerous; endothelial cells, eosinophiles, mast cells, and erythrocytes may be seen.

Method of Counting the Cells.[1]—A saturated aqueous solution of methyl violet (5B) is drawn up in the tube of a 1:100 blood pipette, until it has filled four of the decimal divisions on the capillary tube, and then the pipette is filled with the fresh spinal fluid, which should have been well shaken just before making the dilution to insure a uniform suspension of the cells. The leukocytes are stained violet. The pipette is shaken three minutes, and the count is then made with the blood-counting chamber, observing

[1] Rous, F. P. "Clinical studies of the cerebrospinal fluid, with especial reference to pressure, protein-content, and the number and character of the cells." *Amer. Jour. Med. Sci.*, 1907, CXXXIII, 567.

[2] McCampbell, E. F., and Rowland, G. A. "Studies on the clinical diagnosis of general paralysis of the insane." *Jour. Med. Research*, 1910, XXII, 169.

the usual technique for counting blood. Emerson [1] recommends the use of the leukocyte pipette. The capillary tube is filled to the mark 0.5 with Unna's polychrome methylene blue, then to the mark 11 (or 21) with the fresh cerebrospinal fluid. With this procedure more cells are counted.

At times, in performing lumbar puncture, blood becomes mixed with the cerebrospinal fluid, the result being that the blood leukocytes raise the count of the cells of the cerebrospinal fluid. In such case the red cells in the fluid are counted, then both red and white cell counts of the blood are made. From the latter one obtains the relative number of red and white cells which have been introduced into the cerebrospinal fluid, and the count of the latter may be corrected. In making such correction it is essential that there shall have been no laking of the erythrocytes in the cerebrospinal fluid.

Differential Count of the Cells.—The fresh fluid is centrifugalized, and the sediment is transferred with a pipette to clean glass slides, on which it is spread. The dried films are fixed and stained, usually with a Romanowsky stain. A differential count of the cells is then made [2] (see p. 296).

Bacteriology of the Cerebrospinal Fluid.—Culture media, preferably blood agar, should be inoculated with the fluid. (For further details see the works on bacteriology.) Smears are also made and examined. The *Micrococcus intracellularis meningitidis*, *Diplococcus pneumoniæ*, and *Bacillus influenzæ* are among the most important organisms encountered.

Bacillus tuberculosis is demonstrable in the great majority of instances of tuberculous meningitis. Several cu-

[1] Emerson, C. P. "Clinical Diagnosis." 3rd Edition, 1911, p. 700.

[2] Szecsi, S. "Neue Beiträge zur Cytologie des Liquor cerebrospinalis: Ueber Art und Herkunft der Zellen." *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 1911, VI, 537.

bic centimeters of the fluid are placed in a sterile test tube, which is put in the refrigerator for 12 to 24 hours. A very delicate, filmy clot forms. This enmeshes the majority of the tubercle bacilli. The clot is then transferred to a clean slide, spread out into a thin layer with needles, air-dried, and fixed with heat. The specimen is then stained by the Ziehl-Neelsen method for tubercle bacilli. With this method Hemenway [1] has demonstrated the organisms in a large series of cases.

A second method of searching for the tubercle bacillus, which gives less constant results, is to centrifugalize the fluid at high speed until the sediment is thrown down (about 2,000 revolutions for ½ hour); it is then transferred to slides and examined in the usual manner for tubercle bacilli.

In case of negative findings, several cubic centimeters of the fluid may be injected into the peritoneal cavity of a guinea-pig (see p. 317).

Globulin Content.—Globulin is the most important protein of the cerebrospinal fluid. It is often present in increased quantity in disease, and may be detected by the following tests. Fluids with an admixture of blood cannot be used, since the serum globulin vitiates the tests.

(1) METHOD OF NOGUCHI.[2]— To 0.1 c. c. of spinal fluid add 0.5 c. c. of a 10 per cent. solution of *pure* butyric acid in 0.9 per cent. sodium chlorid and boil briefly over the flame. Then add quickly 0.1 c. c. of $\frac{N}{1}$ sodium hydroxid and again

[1] Hemenway, J. "The constant presence of tubercle bacilli in the cerebrospinal fluid of tuberculous meningitis." *Amer. Jour. Dis. Child.*, 1911, I, 37.

[2] Noguchi, H. "The relation of protein, lipoids, and salts to the Wassermann reaction." *Jour. Exp. Med.*, 1909, XI, 84. Noguchi, H., and Moore, J. W. "The butyric acid test for syphilis in the diagnosis of metasyphilitic and other nervous disorders." *Ibid.*, 1909, XI, 604. *See also* Strouse, S. "The diagnostic value of the butyric acid test (Noguchi) in the cerebrospinal fluid." *Jour. A. M. A.*, 1911, LVI, 1171.

boil for a few seconds. With an increase of globulin a coarse granular or flocculent precipitate appears, usually within 10 to 20 minutes. If the precipitate does not appear within this time, the test tube is set aside and observed at the end of three hours. Normal fluids or those in which globulin is not increased give rise to a slight and uniform opalescence only, and no coarse precipitate forms, even after several hours. In case of an ambiguous reaction, Noguchi advises a repetition of the test with 0.2 c. c. of spinal fluid.

(2) METHOD OF ROSS AND JONES.[1]—Two c. c. of a saturated aqueous solution of ammonium sulphate are placed in a test tube, and 1 c. c. of the spinal fluid is gently run onto the surface of it, while the tube is inclined, so as to form a layer above the ammonium sulphate. The formation of a thin, grayish-white ring at the line of contact of the two fluids constitutes a positive reaction. The precipitate should form within three minutes. Within one-half hour it may be observed that the surface of the ring shows a delicate mesh appearance resembling a fine cobweb. The ring should be looked for with indirect illumination, the tube being held against a dark background with the eye at a right angle to the source of light. It is essential that the ammonium sulphate be quite neutral, not ac[illegible] and that the solution be saturated.

The Wassermann Reaction in Cerebrospinal Fluid.—In syphilitic and metasyphilitic disease of the central nervous system the fluid obtained at lumbar puncture may yield a positive Wassermann reaction.

[1] Ross, G. W., and Jones, E. "On the use of certain new chemical tests in the diagnosis of general paralysis and tabes." *British Med. Jour.*, 1909, I, 1111.

INDEX

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V

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M87 Clinical laboratory me-
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